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Investigating the PWS gene *MAGEL2* as a Candidate Gene for Obesity

by

Megan Anne O'Neill 

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ABSTRACT

Prader-Willi Syndrome (PWS) is a disorder involving a hypothalamic defect. *MAGEL2* is one gene inactivated in individuals with PWS, and is most highly expressed in the hypothalamus. We hypothesize that a lack of *MAGEL2* expression contributes to features of PWS, specifically hyperphagia, obesity and excessive daytime sleepiness. DNA from children with severe obesity and hyperphagia were screened for mutations in *MAGEL2*. While no detrimental mutations were identified, a previously unidentified polymorphism was identified. This change was confirmed to be a polymorphism because the same sequence change was identified in a population of control DNA samples. A *Magel2*-knock out mouse model was studied for characteristics of PWS. These mice appear more prone to obesity. The male knock-out mice are subfertile; a possible side effect of obesity or a direct problem resulting from the loss of *Magel2* expression. The results in this thesis outline the consequences of the loss of *Magel2* expression.

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LIST OF NOMENCLATURE AND ABBREVIATIONS

ADHD - Attention deficit hyperactivity disorder

Arc - Arcuate nucleus

AVP - Vasopressin

BAC - Bacterial artificial chromosome

BMI - Body mass index

bp - base pair

BP - Break Point

C57Bl/6 - C57 BLACK6 mouse strain, originally derived in 1921

CNS - Central nervous system

CSF - Cerebrospinal fluid

DD - Dark-Dark (complete darkness)

DHPLC - Denaturing high performance liquid chromatography

DNA - Deoxyribonucleic acid

EDS - Excessive daytime sleepiness

FISH - Fluorescent *in situ* hybridization

fMRI - Functional magnetic resonance imaging

GH - Growth hormone

GHRH - Growth hormone releasing hormone

GnRH - Gonadatropin releasing hormone

HLA - Human leukocyte antigen

IC - Imprinting center

IM - Imprinting mutation

IQ - Intelligence quotient

IR - Immunoreactive

IRD - Interspersed repeat domain

Kb - kilo base

LD - Light-Dark (12 hours light followed by 12 hours dark)

MAGE - Melanoma-(associated) antigen (gene family)

MAGEL2 - MAGE like-2 gene

mf - Mutant female

MHD - MAGE homology domain

mm - Mutant male

MRI - Magnetic resonance imaging

NDN - Necdin; Neurally differentiated embryonic carcinoma-cell derived factor

NPY - Neuropeptide Y

OCD - Obsessive-compulsive disorder

OFC - Orbitofrontal cortex

ORF - Open reading frame

OSA - Obstructive sleep apnea

PCR - Polymerase chain reaction

PDD - Pervasive developmental disorder

PFC - Prefrontal cortex

PPBS - Posterior pituitary bright spot

PVN - Paraventricular nucleus (of the hypothalamus)

PWS - Prader-Willi Syndrome

REM - Rapid eye movement

RFLP - Restriction fragment length polymorphism

RGCs - Retinal ganglion cells

SCN - Suprachiasmatic nucleus

SEM - Standard error of the mean

snoRNAs - Small nucleolar RNAs (RNA: ribonucleic acid)

SON - Supraoptic nucleus

UPD - Uniparental disomy

VMH - Ventromedial hypothalamus

wf - Wild type female

wm - Wild type male

WT - Wild type

CHAPTER 1 ★ INTRODUCTION

PRADER-WILLI SYNDROME

Prader-Willi syndrome (PWS) is a neurobehavioral disorder characterized by obesity with hyperphagia, short stature, small hands and feet (acromicria), and hypogonadism, with developmental and behavioural abnormalities. The complex phenotype of this syndrome results from a developmental defect in the hypothalamus (1-3). In 1956, Drs Prader, Labhart, and Willi published the description of Prader-Labhart-Willi syndrome , which is now known as Prader-Willi syndrome (4). When PWS was first described, the syndrome could only be diagnosed based on external features (5). Since then, the fundamental cause of PWS has become clear. PWS is a sporadic genetic disorder that is caused by the loss of activity of a cluster of genes on chromosome 15. Because the physical features that compose PWS can vary greatly, a definitive diagnosis is obtained through genetic testing (5). Infants with PWS are characterized by neonatal hypotonia and failure to thrive, which often requires the use of a gastric feeding tube (3, 5). Children with PWS change dramatically between the ages of 2 and 3 years old. As well as being mostly sedentary due to their lack of muscle tone, they also become hyperphagic, which leads to excessive weight gain and obesity. The hyperphagia will persist throughout the life time of the PWS individuals. The other major characteristics consist of short stature, acromicria, hypogonadism, behavioural problems, and global developmental delay (5-7).

CLINICAL FEATURES OF PWS

EPIDEMIOLOGY

PWS occurs in all ethnicities and affects males and females equally (8, 9). The estimated prevalence of PWS among live births varies greatly in the current literature. The approximate frequency of PWS is estimated between 1 in 8,000 and 1 in 25,000 live births, with a generally accepted frequency of 1 in 15,000 (6, 8, 9). It is estimated that the population prevalence is 1 in 50,000 (10). The lesser frequency among the population is due to a number of missed diagnoses in the older population, a higher neonatal mortality rate in the past and an increase in mortality with age (11).

The life expectancy of individuals with PWS can vary greatly, depending on the life style of the given individual, and there is no definable natural life span in the current literature (6). When weight and diet are controlled, individuals with PWS generally have the same life expectancy of other mildly retarded individuals (8, 12). However, infants, children, and adults with PWS have an increased frequency for sudden unexpected death, as compared to the general population (13). Because there are many complications in the infantile period, there is an increase in mortality, which then distorts the average life expectancy of individuals with PWS. The cause of death in infants with PWS is usually attributed to febrile illness and respiratory difficulties (13). In children and adults with PWS, death is usually attributed to obesity and obesity-related complications (12, 13).

THE PWS NEONATE

The complexity of PWS is demonstrated by multiple characteristics present in both the neonatal period and the childhood to adulthood period (6, 12). Even before the neonatal period, there are notable differences in the fetal gestational period of PWS individuals. For pregnancy and delivery of a PWS infant, 76% have a reduction in fetal activity compared to a pregnancy with a normal infant (6). Approximately 25% of PWS neonates present in the breech position, which could be an extension of the reduction in fetal movement (6). There is a slight reduction in the average weight of PWS infants (6). Over 40% of PWS infants are non-term deliveries. These infants are either born 2 weeks prior to, or 2 weeks later than the expected delivery date (6). Hypothalamic dysfunction is a well supported etiology for PWS. A fetal hypothalamus that is functioning correctly is crucial for a normal birth timing, which may explain the high frequency of premature and postmature deliveries of individuals with PWS (8).

The most profound presentation in the newborn infant with PWS is severe hypotonia (6, 14). The PWS neonate will have hypotonia, with poor muscle tone, and are often called a "floppy-baby" in layman's terms. The hypotonia will improve with age, usually when the infant is around 8 to 11 months old. Infants with PWS tend to sit at 11-12 months of age, and are generally crawling at 15-16 months (6, 12, 15). The presentation of hypotonia is not specific to PWS, and these infants can often be misdiagnosed early on in life. In conjunction with hypotonia, there are numerous other diagnostic criteria that are needed to suggest a diagnosis of PWS.

Failure to thrive is one of the most serious of symptoms in infancy. The other very serious symptom is respiratory abnormalities, including sleep-related central and obstructive apneas (6). PWS infants commonly have difficulties in feeding, leading to failure to thrive, with a lack of weight gain that can put the infant into the 3rd or less percentile. Many of these infants show a poor suck reflex (6, 8, 9, 12, 15). To treat the feeding difficulties and failure to thrive, a gastric feeding tube is often used, and can be needed for months (6, 10).

PWS infants exhibit excessive sleepiness and can often be difficult to arouse (6, 14). These infants rarely cry, and when they do, their cries are very weak (6). PWS infants do not move as much as normal infants, likely due to hypotonia and poor muscle strength, and because they sleep much more than normal infants.

Hypogonadism and hypogenitalism are major criteria in the diagnosis of PWS, and can be detected at birth. In males, hypogonadism presents as cryptorchidism, small testes, decreased scrotal rugae, with a hypoplastic penis. Females tend to present with absent or severe hypoplasia of the labia minora and/or clitoris (6, 8-10, 12, 15). This is discussed in more detail in the next section.

Temperature instability occurs in almost all PWS individuals, but is still only considered a supportive finding, rather than a major or minor criterion for diagnosing PWS. Predominantly, infants and young children with PWS present with thermolability, although it is thought that this dysregulation of temperature is present through the life of

the individual. Older individuals with PWS have a general alteration in their temperature sensitivity. The fluctuation and poor control of body temperature is credited to a malfunction of the hypothalamus (6, 8, 13, 14, 16, 17). Bradycardia and tachycardia have been reported in individuals with PWS. Notably, the changes in heart rate are concurrent with fever, hypertension, and in older individuals, with obesity (13, 14).

Õiglane-Shlik *et al* recently published a report on the clinical phenotype of the PWS neonate. The authors reported findings not commonly documented, due to their benign nature. All five PWS neonates examined demonstrated acrocyanosis, and three also showed marked mottling of the skin. Acrocyanosis consists of symmetrical cyanosis of the extremities, with persistent, uneven mottled blue or red discolouration of the skin of the digits, wrists and ankles with profuse sweating and coldness of the digits (14, 18). It seems quite likely that severe hypotonia and thermolability are strong contributing factors in the presentation of acrocyanosis.

PHYSICAL CHARACTERISTICS OF PWS

Individuals with PWS have distinctive facial characteristics. These features are a prominent but narrow forehead, a small upturned nose, a long, narrow looking head (dolichocephaly), almond-shaped eyes with strabismus, down-turned corners of the mouth (making the mouth look triangular), and a thin upper lip (6, 9, 12, 14, 15). Many of the distinctive dysmorphic facial features, such as the nose, resemble those due to midline central nervous system defects. However, it is thought that other features, such as the triangular shape of the mouth, are related to the hypotonia and poor musculature in

these individuals (6). In addition to almond-shaped eyes, many individuals with PWS present with strabismus, both esotropia (deviation of the eye toward the nose) and exotropia (deviation of the eye toward the temple). These ocular findings may be related to hypotonic ocular muscles (6). In addition to strabismus, some PWS individuals are myopic, with the mean level of myopia being greater than the norm for their age, sex and BMI matched control individuals. It has also been noted that the myopia tends to be high in relation to other family members (6, 10, 19). Ocular hypopigmentation is another ophthalmologic disorder that may be present in individuals with PWS (6).

In addition to ocular hypopigmentation, general hypopigmentation is seen in a large proportion of individuals with PWS. The hypopigmentation is correlated to PWS individuals with a deletion on chromosome 15 (genetic causes of PWS are discussed in a later section). The *P* gene (OCA2) is located within the region of chromosome most commonly deleted from the paternal chromosome 15. It is believed that deletion of this paternal allele, making *P* hemizygous, results in the hypopigmentation seen in many of the individuals with PWS (6, 10, 12, 15).

Hypogonadism and under development of the genitalia are major criteria in the presentation of PWS. Males will present with small penis, small testes, and cryptorchidism, and surgery is often performed shortly after birth to correct the cryptorchidism. In addition to surgery, some males are given hormonal treatment, as the level of circulating testosterone in their blood is abnormally low. Even at the age of normal pubertal onset, most males with PWS will only show slight pubertal development.

Therefore additional testosterone will induce secondary sex characteristics and penile growth, which can be very important for the psychosocial well-being of the individual. Testosterone is also know to be beneficial in PWS males; it helps preserve bone mass, preventing osteoporosis, and it also increases muscle mass and strength (6). Despite testosterone treatment, males with PWS are infertile (5, 6, 8-10, 12, 15). Testicular biopsy revealed the presence of Sertoli cells, but the absence of germinal cells (spermatogonia) (6). Females present with hypogenitalism that is much less complex, and surgery is generally not required. Some PWS females will go through a relatively normal pubertal stage, while in other PWS females, puberty is very delayed and incomplete. The majority of PWS females will not reach full sexual maturity. There is evidence of phenotypic puberty, as the breasts will develop, but menorrhea is often late or does not occur in females with PWS. If a PWS female does menstruate, it is often irregular, and many menstruating females with PWS will encounter menopause early. There are rare cases of PWS females being fertile, but generally females with PWS are considered infertile (5, 6, 8-10, 12, 15). In adolescents and adults with PWS, there are low levels of GnRH (gonadatropin releasing hormone), suggesting a hypothalamic-pituitary origin for the hypogonadotropic hypogonadism (6).

Small hands and feet in individuals with PWS was one of the first characteristics used as an identifiable feature in diagnosing PWS. Small hands and feet (acromicria) first become recognizable during mid-childhood (6). PWS individuals have small hands with thin, tapered fingers. Despite obesity, there is relatively little subcutaneous fat stored in the hands. The feet are smaller in relationship to hand size; foot size is less than the tenth

percentile and hands are less than the twenty-fifth percentile, for height and age (6, 8, 10). As with the hands, the feet also contain relatively little subcutaneous fat, and in obese PWS individuals it often appears that the fat abruptly stops at the wrists and ankles (6).

Short stature is a cardinal feature of PWS. Without medical intervention, the average height of a PWS male is 155cm (61 inches) and 147 cm (58 inches) for PWS females. The pathogenesis of short stature involves growth hormone (GH) deficiency, which is evidenced by low or deficient serum GH levels in children with PWS. GH is released from the anterior pituitary by specialized cells. These cells are stimulated to release GH through input signals that come from the arcuate nucleus of the hypothalamus (6). Because PWS involves hypothalamic dysfunction, it seems reasonable to assume that the short stature seen in PWS is due to lack of signalling to release GH, rather than a dysfunction in the reception of the GH signal telling the body to grow. This has led to the use of exogenous GH as a treatment of PWS. It is currently the only medication to receive regulatory approval for treatment of PWS children (6). GH treatment not only restores linear growth, but also improves lean body mass, while decreasing the fat mass, and increases bone density (6). The use of GH therapy in PWS children is still a little controversial. This controversy stems from reports in the early years of GH replacement therapy, which suggested that GH therapy had increased the risk of death as it created or exacerbated respiratory problems, especially sleep related apneas. This controversy has somewhat subsided as more recent reports argue that there is no indication that GH will increase the risk of death, but strongly suggest a need for careful determination of the dose of GH and careful monitoring of patient's respiratory conditions, especially if there

are known pre-existing respiratory difficulties (6, 17). Presently, controversy remains surrounding the idea that GH therapy should be continued into and possibly throughout adulthood. Many of the clinical features observed in adults with PWS (e.g. osteoporosis, increased body fat, cardiovascular risks) are also present in non-PWS GH-deficient adults. The use of GH for non-PWS GH-deficient individuals is beneficial; it improves body composition, lipid profiles, bone density, and perhaps most importantly, the therapy has improved quality of life (6). It seems reasonable to accept the notion that GH replacement therapy, even in minimal doses, will have the same beneficial results in PWS adults.

PWS children are more likely to be diagnosed with scoliosis than other children. The reason for this increased risk of scoliosis is still unclear, although it is thought that the scoliosis in PWS comes under the category of neuromuscular scoliosis (5, 6). The type of scoliosis that presents in PWS is likely myopathic neuromuscular scoliosis, due to hypotonia of the paravertebral muscles (6). Scoliosis in PWS is often noted in individuals who are not overweight, indicating that obesity is not a contributing factor to the pathogenesis of scoliosis (6). Another bone abnormality, osteoporosis, is considered a suggestive or additional criteria in the diagnosis of PWS. In individuals with PWS, osteoporosis is attributed to loss of bone mineral due to lack of endogenous growth hormone, nutritional deficiencies (specifically calcium), and lack of neuromuscular stimulation (exercise) (6).

It has been known for a long period of time that the majority of individuals with PWS have some form of respiratory difficulty. Abnormalities in daytime/awake breathing are restrictive lung disease, and an abnormal response to hypercapnia and hypoxia (6, 16). The risk of developing restrictive lung disease is increased in individuals with PWS due to obesity and the increased incidence of scoliosis. Normal breathing can be broken down into three steps: (1) exchange of air between the lungs and atmosphere via thoracic movement and ventilation, (2) exchange of O_2, CO_2 and other gases across the alveolar membranes, and (3) circulation, delivery and exchange of gases in the tissues (6). The purpose of breathing is to maintain tissue oxygen levels and CO_2 homeostasis. Respiratory abnormalities in PWS are exclusively linked to abnormal thoracic movement and ventilation, as there are no abnormalities in the structure of the lung tissue and no reported abnormalities in O_2/CO_2 transport (6). In PWS, restrictive lung disease is due to thoracic muscle weakness and respiratory muscle hypotonia as the cause of the restriction defect. Obesity is thought to *compound* the thoracic muscle weakness and hypotonia leading to restrictive lung disease, not cause it. This is evidenced by observed respiratory problems without excess body weight, as in neonates with PWS (6, 16). The abnormal response to hypercapnia and hypoxia seen in individuals with PWS are independent of the degree of obesity. The exact cause of this reduced response is still unclear, but there is promising evidence from animal studies that the hypothalamus plays a role in modulating the ventilatory response to hypoxia and hypercapnia (6, 16).

Many PWS individuals have abnormal sleep and sleep-disordered breathing. Central, obstructive and combined apneas described in PWS are characteristic of neuromuscular

weakness of the respiratory musculature (6). Individuals with PWS are monitored closely for obstructive sleep apnea (OSA), as it is carries a high risk of death. PWS individuals are at risk of developing OSA for multiple reasons, including obesity, facial dysmorphism (small mouth and a small naso- and/or oro-pharynx diameter), thick and sticky saliva, and hypotonia (5, 6, 16).

Hypersomnia and excessive daytime sleepiness (EDS) are common features of PWS, occurring in 70-95% of PWS individuals (10, 16). Sleep abnormalities have been documented in individuals with PWS at all ages with and without obesity. EDS in some PWS individuals can be severe enough that they could be medically diagnosed with narcolepsy (6, 10, 16). EDS is present despite increased *quantity* of nocturnal sleep in patients with PWS. As well, the quality of sleep is usually excellent, with less night-time awakenings and increased sleep time compared to normal individuals, and an increased proportion of slow-wave sleep (16). Therefore, the *quality* and *quantity* of sleep are not contributing to the EDS seen in PWS, suggesting that EDS is a primary feature of PWS. Also, there is no evidence that sleep-disordered breathing and EDS are related, again supporting the notion that EDS in PWS is a primary abnormality and is not secondary to sleep disturbance caused by sleep-disordered breathing (16). PWS individuals are quite likely to fall asleep in quiet, boring environments. Treatment of EDS includes adjustments in the daily routine so as to avoid such sleep-inducing environments, as well as behavioural incentives to stay awake (6, 16). Individuals with PWS exhibit abnormalities of REM sleep (rapid eye movement), which is independent of obesity and of PWS genotype (deletion vs. UPD; see section Genetic causes of PWS). The timing of

the onset of REM sleep is disrupted in PWS, such that there is a shorter REM latency and REM sleep occurring earlier than in normal subjects (6, 16). Some individuals have REM sleep occurring at the beginning of sleep, which is a feature of narcolepsy. It has been reported that approximately 16% of PWS patients also exhibit cataplexy, another feature of narcolepsy. However, the HLA (human leukocyte antigen) subtypes associated with narcolepsy have not been identified in any of the PWS patients studied. It is therefore assumed that the similarity between narcolepsy and PWS, regarding the abnormalities in REM sleep, are due to the hypothalamus and its role in the maintenance of arousal (6, 16). Interestingly, the sleep-disordered breathing noted in PWS individuals occurs mostly during REM sleep (6, 20).

Obesity is the cardinal feature of PWS. PWS individuals have childhood onset obesity, which can continue to diminish the heath of the individual if it is not controlled (6, 10). The childhood obesity is accompanied by hyperphagia (over eating), starting between the ages of 1 and 3 years old. The obesity seen in individuals with PWS differs from the obesity seen in non-PWS obese individuals. The distribution of subcutaneous fat is around the thighs, upper arms, abdomen and buttocks, while the extremities are usually relatively slim. In non-PWS obese individuals, the distribution of subcutaneous fat is even over the entirety of the body (6, 15, 19). If dietary constraints are not enforced, an adolescent with PWS can easily weigh 250-300 lb. Hyperphagia, persistent hunger, decrease or absence of the perception of satiety, and an uncontrollable appetite are all life long characteristics of PWS, and if an individual's diet is not monitored, these characteristics will lead to morbid obesity. Also, overeating can have an acute effect by

leading to stomach rupture, which is life threatening (6, 15). Obesity in PWS individuals will usually decrease their lifespan to less than 35 years (10). Weight management is achieved through caloric restriction, exercise programs, and is sometimes combined with growth hormone therapy. Typically, an individual with PWS who has lost weight and is looking to maintain their current body weight will only need to consume 60% of the recommended caloric intake for healthy individuals (6, 15). This is likely due to the fact that PWS individuals are more sedentary and therefore burn fewer calories on a daily basis. Exercise regimes are extremely important for individuals with PWS. Exercise will not only help to prevent obesity by burning calories, but it will also help to build muscle mass and muscle strength. Improved muscle strength in children with PWS will encourage them to move about more spontaneously (5).

There are published diagnostic criteria that physicians use to establish if a diagnosis of PWS is likely. These consensus criteria were published in 1993, and recently Gunay-Aygun *et al* have published some proposed changes to the consensus criteria (21, 22). Table 1.1 outlines the consensus criteria proposed in 1993 and Table 1.2 outlines the proposed changes.

Table 1.1 Diagnostic Criteria for Prader-Willi Syndrome. In 1993, Holm *et al* published consensus criteria for diagnosing Prader-Willi Syndrome. Scoring: Major criteria are weighed at one point each. Minor criteria are weighed at one half point. Children 3 years of age or younger: 5 points are required for diagnosis, 4 of which should come from the major group. Children 3 years of age to adulthood: Total score of 8 is necessary for the diagnosis. Major criteria must compromise 5 or more points of the total score. Consensus criteria from Holm, V. A. *et al*, "Prader-Willi-Syndrome: Consensus Diagnostic Criteria". *Pediatrics*, Vol. 91(2), pg 398-402.

Table 1.1 Diagnostic Criteria for Prader-Willi Syndrome
MAJOR CRITERIA
1. Neonatal and infantile central hypotonia with poor suck, gradually improving with age
2. Feeding problems in infancy with need for special feeding techniques and poor weight gain/failure to thrive
3. Excessive or rapid weight gain on weight-for-length chart (excessive is defined as crossing two centile channesl) after 12 months but before 6 years of age; central obesity in the absence of intervention
4. Characteristic facial features with dolichocephaly in infancy, narrow face or bifrontal diameter, almond-shaped eyes, small-appearing mouth with thin upper lip, down-turned corners of the mouth (3 or more required)
5. Hypogonadism - with any of the following, depending on age: a. Genital hypoplasia (male: scrotal hypoplasis, cryptochidism, small penis and/or testes for age {<5th percentile}; female: absence or severe hypoplasis of labia minora and/or clitoris) b. Delayed or incomplete gonadal maturation with delayed pubertal signs in the absence of intervention after 16 years of age (male: small gonads, decreased facial and body hair, lack of voice change; female: amenorrhea/oligomenorrhea after age 16)
6. Global developmental delay in a child younger than 6 years of age; mild to moderate mental retardation or learning problems in older children
7. Hyperphagia/food foraging/obsession with food
8. Deletion 5q11-13 on high resolution (>650 bands) or other cytogenetic/molecular abnormality of the Prader-Willi chromosome region, including maternal disomy
MINOR CRITERIA
1. Decreased fetal movement or infantile lethargy or weak cry in infancy, improving with age
2. Characteristic behavior problems - temper tantrums, violent out burst and obsessive/compulsive behavior; tendency to be argumentative, oppositional, rigid, manipulative, possessive, and stubborn; perseverating, stealing, and lying (5 or more of these symptoms required)
3. Sleep disturbance or sleep apnea
4. Short stature for genetic background by age 15 (in the absence of growth hormone intervention)
5. Hypopigmentation - fair skin and hair compared to family
6. Small hands (<25th percentile) and/or feet (<10th percentile) for height and age
7. Narrow hands with straight ulnar border
8. Eye abnormalities (esotropia, myopia)
9. Thick viscous saliva with crusting at corners of the mouth
10. Speech articulation defects
11. Skin picking
SUPPORTIVE FINDINGS (increase the certainty of diagnosis but are not scored)
1. High pain threshold
2. Decreased vomiting
3. Temperature instability in infancy or altered temperature sensitivity in older children and adults
4. Scoliosis and/or kyphosis
5. Early adrenarche
6. Osteoporosis
7. Unusual skill with jigsaw puzzles
8. Normal neuromuscular studies

Table 1.2 Suggested New Criteria to Warrant DNA Testing for PWS. With time, there has been an increase in the availability and feasibility of genetic testing. Therefore, rather than having a table of diagnostic criteria, that is scored subjectively, this new criteria is to suggest genetic testing which is an objective test. New criteria from Gunay-Agun, M. *et al*, "The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria". *Pediatrics*, Vol. 108(5), E92.

Table 1.2 Suggested New Criteria to Warrant DNA Testing for PWS	
Age At Assessment	**Features Sufficient to Prompt DNA Testing**
Birth to 2 years	1. Hypotonia with poor suck
2 years - 6 years	1. Hypotonia with history of poor suck 2.Global developmental delay
6 years - 12 years	1. History of hypotonia with poor suck (hypotonia often persists) 2. Global developmental delay 3. Excessive eating (hyperphagia; obsession with food) with central obesity if uncontrolled
13 years through adulthood	1. Cognitive impairment; usually mild mental retardation 2. Excessive eating (hyperphagia; obsession with food) with central obesity if uncontrolled 3. Hypothalamic hypogonadism and/or typical behavior problems (including temper tantrums and obesessive-compulsive features)

METABOLIC AND ENDOCRINE FEATURES OF PWS

Ghrelin is a peptide that is released from cells in the stomach, with plasma levels peaking when fasting and before the start of a meal. The plasma level of ghrelin declines after consumption of a meal. The normal function of ghrelin is not defined completely, but the literature strongly supports the notion that ghrelin stimulates food intake acutely (6, 10, 15, 23). This is evidenced by studies on rodents and humans in response to exogenously administered ghrelin. Chronic administration of ghrelin to rodents causes an increased appetite and obesity (6, 10). Adults, who have fasted overnight, are administered exogenous ghrelin to achieve serum ghrelin concentrations of approximately twice the normal fasting level. These individuals demonstrated a potent increase in appetite and

food intake (6, 10, 23). The current literature states that there is an increase of serum ghrelin levels in PWS (6, 10, 23). In healthy weight individuals and non-PWS obese individuals, there is an inverse correlation between BMI and ghrelin levels. However, in PWS individuals, there is no correlation between BMI and ghrelin levels. Because of the increased ghrelin in PWS individuals, regardless of their BMI, it is speculated that ghrelin may be an orexigenic factor contributing to the markedly increased appetite and obesity seen in PWS (10, 23).

Leptin and its receptor are implicated in obesity, as mutations identified in those genes cause non-PWS hyperphagia, obesity, and hypogonadism. This then raised the possibility that defects in leptin pathways in the brain may explain these phenotypes in PWS patients (24). Leptin is produced by adipocytes (fat cells), and signals the brain that there is adequate energy storage in the body. This signals occurs as the circulating leptin interacts with brain pathways, particularly in the hypothalamus, to reduce food intake and control energy expenditure and body weight (24). Serum levels of leptin directly correlate with the amount of body fat (6). This is also true in PWS individuals, who usually have higher leptin levels due to their increased body fat. There have been no mutations in the leptin gene (*OB*) or the leptin receptor gene (*OBRb*) identified in individuals with PWS (6). Therefore, it appears that the leptin pathway is normal in PWS individuals.

Insulin is secreted by the pancreas in response to high blood sugar levels and induces hypoglycemia (18). Defective insulin secretion or insulin resistance causes diabetes

mellitus. Diabetes mellitus is strongly correlated with obesity, and the onset is generally in adolescence or adulthood (18). Many obese individuals with PWS will develop glucose intolerance and Type 2 diabetes mellitus (non-insulin dependent). Interestingly, compared to non-PWS obese individuals of similar weight, PWS individuals have a lower insulin resistance and a higher sensitivity to insulin (6).

NEUROLOGICAL FEATURES OF PWS

There are recent studies on specific areas of the brain, as well as localized brain activity in live patients after a specific treatment, that indicate there may be subtle, but likely significant, differences in the brains of PWS individuals. Anatomical and histopathological studies on post-mortem brains from PWS individuals have shown enlarged lateral ventricle volume, cerebellar dentate nucleus abnormalities, neuron cell layer disorganization and cell loss, and frontal cortical atrophy (25). Using the hypothalami from five deceased PWS patients, Swaab *et at* investigated histopathology with conventional thionine staining and immunocytochemical staining for oxytocin and vasopressin (26). The parvocellular oxytocin neurons of the hypothalamic paraventricular nucleus (PVN) are involved in inhibiting food intake, therefore making this a reasonable area to investigate in PWS individuals. In PWS patients, it was found that the volume of the PVN was reduced by 28% and the total cell number was 38% lower. There was a highly significant decrease in the number of oxytocin-expressing neurons in PWS patients (42%) (26, 27). Also, the volume of the PVN containing the oxytocin-expressing neurons was 54% lower in PWS (26, 27). The authors found that the

number of vasopressin (AVP)-expressing neurons in the paraventricular nucleus were not significantly different from matched controls. These findings indicate that PWS is a disorder of hypothalamic development, and supports the notion that oxytocin neurons in the PVN function physiologically as "satiety neurons" (26, 27).

To analyse satiety dysfunction in PWS, Shapira *et al* used functional magnetic resonance imaging (fMRI) to measure brain activity after ingestion of a glucose solution. Three adults with PWS fasted overnight and drank a 75g dextrose solution prior to fMRI scans. Brain activity levels were measured during and after the glucose ingestion. Following the glucose ingestion, there was a significant delay in hypothalamic activation, as well as in other brain regions associated with satiety. These other brain regions include the insula, ventromedial prefrontal cortex, and nucleus accumbens. The mean latency (time between glucose administration and hypothalamic activity), in PWS individuals was 24 minutes. This is significantly different from non-PWS, obese individuals and lean individuals who have mean latency times of 15 minutes and 10 minutes, respectively (28, 29). The study identified the insular cortex, the prefrontal cortex, the ventral basal ganglia, and the hypothalamus as consistently being delayed in response to glucose ingestion. These results provide further evidence towards a satiety dysfunction in the CNS of PWS individuals (28, 29).

Limbic and paralimbic regions of the brain, including the orbitofrontal cortex (OFC), medial prefrontal cortex (PFC), insula, and amygdala, are implicated in processes underlying hunger and food motivation. Obese individuals exhibit elevated activity in

their PFC during satiation compared to non-obese individuals. fMRI was used to study the neural mechanisms underlying response to visual food stimuli, before and after eating, in individuals with PWS and a group of healthy controls (25). Post meal, the PWS group showed hyperfunction in limbic and paralimbic regions that drive eating behaviour (for example, the amygdala), and in regions that suppress food intake (for example, the medial PFC). The results provide evidence that PWS individuals display different patterns of neural activation in response to food stimuli compared with a group of non-obese controls. This suggests a distinct neural mechanism associated with hyperphagia in PWS. The results in this study and the results reported by Shapira *et al* support the idea that hyperphagia in PWS results from lack of satiation resulting from neural dysfunction (25, 28).

Narcolepsy is associated with lowered cerebrospinal fluid (CSF) levels of hypocretin (orexin), resulting from a loss of hypocretin neurons in the perifornical area of the hypothalamus. PWS individuals exhibit excessive daytime sleepiness (EDS), which resembles many features of narcolepsy. Therefore, it was hypothesized that the narcolepsy-like symptoms in PWS are caused by a decline in the number of hypocretin neurons (30). Using immunocytochemistry, the number of hypocretin-containing neurons in the lateral hypothalamus was determined using post-mortem samples of adult and infant PWS individuals, as well as in matched controls (30). The number of hypocretin immunoreactive (IR) neurons in post-mortem material in PWS patients was not different from that in controls. However, it was not known if these PWS patients had narcoleptic features. Therefore, it is possible that PWS individuals with clear

narcoleptic/EDS features may have a lower number of hypocretin IR cells, indicative of a disruption in the hypothalamic hypocretin system (30).

Individuals with PWS are deficient in growth hormone (31). Neurosecretory cells of the hypothalamus secrete growth hormone releasing hormone (GHRH) into portal capillaries located in the median eminence, which it at the base of the hypothalamus. The GHRH is transported to target endocrine cells in the anterior pituitary, signalling these cells to produce and release GH (32). It was hypothesized that the GH deficiency seen in PWS was due to a reduction in the gross morphological size of the anterior pituitary gland (31). Fifteen GH-deficient PWS patients were studied using MRI to determine the size of the pituitary. There was no statistically significant difference found in the height of the anterior pituitary gland in PWS patients, compared to healthy or isolated GH-deficient individuals (31). Interestingly, three patients (3 of 15; 20%) produced MRI images showing complete absence of the posterior pituitary bright spot (PPBS), and a fourth patient demonstrated a small PPBS (31). The PPBS is a direct reflection of hypothalamic function, therefore indicating distinctive hypothalamic dysfunction in these patients.

INTELLECTUAL AND BEHAVIORAL ABNORMALITIES IN PWS

Intellectual disability was a defining characteristic when PWS was first described in 1956 (6, 12). The intellectual development in PWS children is retarded from infancy (5). The majority of PWS individuals will have an IQ in the borderline to normal range, with an average score of 70 (5, 6). Even in PWS individuals with an IQ in the normal range,

there are still distinct cognitive dysfunctions. Speech and gross motor skills are particularly slow to develop in PWS children (5). Many individuals with PWS have difficulty learning to talk, but most understand language much better than they themselves can express (5, 6). Overall, there are four cognitive deficits: (1) depressed general cognitive functioning or IQ, (2) general processing deficits including short-term memory, (3) language processing deficits, and (4) an inability to meld diverse and detailed internal information into relevant higher-order abstract and metacognitive concepts that guide behaviour over the long term (6). Most PWS individuals have severe learning disabilities (5, 9). Wigren and Hansen published a report on ADHD symptoms in PWS, and found that 25% of PWS patients have ADHD symptoms, and 50% of PWS patients present with insistence on sameness (discussed later in this section). The authors also reported that the ADHD symptoms and insistence on sameness are correlated (33).

There is an interesting phenomenon associated with PWS individuals and their higher-than-normal ability on jigsaw puzzles. Dykens studied jigsaw puzzle skill in children with PWS to assess this phenomenon (34). She was able to show that PWS children performed significantly better than their age- and IQ-matched peers with mental retardation on the jigsaw puzzles. But, more interestingly, she was also able to show that PWS children significantly outperformed typically-developing children on the jigsaw puzzles (34). Visual-motor skills in PWS may contribute to this unusual skill in jigsaw puzzles. It is also thought that their obsessive-compulsive tendencies, and need for order, exactness, and for things to be 'just right', also contribute to the abnormally high skill level in jigsaw puzzles (34).

Concurrent with the transition to hyperphagia, children with PWS exhibit a significant change in behaviour. The behavioural features include tantrums, irritability, mood lability, impulsivity, stubbornness, argumentativeness, difficulties adapting to change in daily routine, high pain threshold, sleep disturbances, inappropriate social behaviour, automutilation (skin picking), anxiety, depression, and obsessive-compulsive features (9, 20, 35, 36). Adolescence and early adulthood are critical stages that show an increase in behavioural and emotional disturbances (35).

Individuals with PWS exhibit behaviours that are repetitive and ritualistic, which can appear obsessive and compulsive (6). Similar symptoms are present in autism, obsessive-compulsive disorder (OCD) and pervasive developmental disorder (PDD). The similarities of repetitive, rigid, and routine behaviour in PWS and autism are interesting because there have been genetic linkage studies that link autism to chromosome 15 (37, 38). PDD is characterized by a reduced ability to understand language, a reduced ability to socially interacted in a normal manner, and limited variety in activities and interests. Individuals with PDD often engage in repetitive activities, and resist changes to the environment or to daily routines (18). PDD is considered a more appropriate way to describe the repetitiveness and rigidity seen in PWS, rather than describing this behaviour as OCD. There are two main differences between the repetitive and rigid behaviour seen in PWS (and PDD) and the same type of behaviour seen in OCD. First, individuals with PWS and PDD have fewer *obsessions* about contamination, sexuality, religion, aggression, and the body, as well as fewer *compulsions* concerning cleaning, checking,

and counting than individuals with true OCD (39). Second, individuals with OCD recognize the urging (of obsessions and compulsions) as a product of their own thoughts and have an understanding of the unnecessary, time consuming, and unreasonable nature of their compulsions. PWS individuals exhibiting obsessive-compulsive symptoms are not aware of their stereotypic behaviour and interests, and make no effort to reduce them. Their behaviour is predominantly associated with specific, restricted and stereotypic interest, such as ordering or arranging things, rather than obsessive thoughts and compulsive behaviour in OCD individuals (39).

There are some reported differences in behaviour characteristics depending on the genotype of the individual with PWS. In general, PWS is caused by the loss of expression of a group of genes on the paternally inherited chromosome 15. This can occur through a deletion, uniparental disomy (UPD) of the maternally inherited chromosome 15, or a mutation in the imprinting center. The literature on this issue is contradictory, therefore only some findings will be discussed here. Butler *et al* have reported on behavioural problems in 3 groups of PWS individuals: (1) Type I deletion, (2) Type II deletion, and (3) UPD. The type I deletion is larger than the type II deletion by approximately 500 kilobases (kb) (Figure 1.3). This 500 kb region contains 4 genes; *NIPA1*, *NIPA2*, *CYFIP1*, and *GCP5* (6, 40). Generally, individuals with type I deletions had poorer behaviour and psychological functioning coupled with reduced independent behaviours, compared to individuals with type II deletions or UPD. This is especially true for incidences and severity of skin picking or self injurious behaviour. PWS individuals with type II deletions seem to fall in the middle, when compared to

behavioural analysis scores of type I deletions and UPD individuals (6, 41). Conversely, a recent study by Descheemaeker *et al* has reported different associations with PWS genetic subtype. The authors report that the UPD/IM (uniparental disomy/imprinting mutation) group of PWS individuals shows a significant increase of symptoms of social dysfunction with peers and of unusual fears. The authors also reported that PWS with IQ > 70 and/or with UPD/IM are at an increased risk of developing more behavioural problems (for example, tantrums, aggression, or stubbornness) than PWS individuals with a low IQ and/or with a deletion (39). As more experiments are done with larger cohorts of PWS individuals of all genetic subtypes, other non-PWS mentally deficient individuals, and healthy control individuals, the differences or lack thereof in behavioural characteristics should become more defined.

NEUROANATOMY AND FUNCTION OF THE HYPOTHALAMUS

The hypothalamus is a complex neurological structure located at the base of the diencephalon. It controls many physiological functions via the autonomic nervous system (ANS) or the endocrine system, or by coordinating both of these systems (32, 42, 43). The hypothalamus and the various regions located within the hypothalamus can be seen in Figure 1.1.

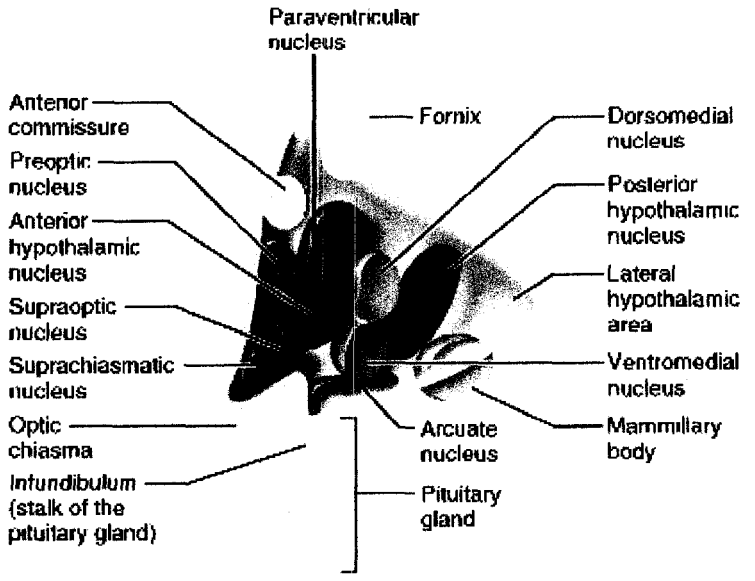


Figure 1.1 Schematic representation of the hypothalamus. The various regions of the hypothalamus are shown as well as the pituitary gland. The image is from http://www.apsu.edu/~thompsonj/Anatomy%20&%20Physiology/2010/2010%20Exam%20Reviews/Exam%205%20Final%20Review/hypothalamus.pituitary.Fig.12.13b.jpg.

The pituitary gland is the master gland of the endocrine system. The hypothalamus controls hormone release from the pituitary through neuronal projections directly from the hypothalamus into the pituitary. Axons from neurosecretory cells, located in the PVN (paraventricular nucleus) or the SON (supraoptic nucleus) in the hypothalamus, project through the infundibulum and form terminals near the posterior lobe of the pituitary. The terminals are the site of hormone (vasopressin and oxytocin) release, which are produced by the hypothalamic cells. There are also hypothalamic projections to the anterior pituitary. Parvocellular neurosecretory neurons in the hypothalamus project through the median eminence to the anterior pituitary. The terminals of these projections release regulatory substances (stimulatory or inhibitory release factors). These factors target endocrine cells in the anterior pituitary signalling these cells to produce or stop producing a specific hormone.

The anterior hypothalamus, preoptic area, and posterior hypothalamus are regions involved in temperature regulation. The anterior region responds directly to cooling or heating, and is involved in promoting heat dissipation. There are thermoreceptor neurons in the anterior hypothalamus and preoptic area that are sensitive to cold, and there is a different set of neurons also in the anterior hypothalamus that are sensitive to heat. The posterior hypothalamus produces a response that leads to heat retention and heat production. There are no thermoreceptor neurons in the posterior hypothalamus, therefore, the heat-generating response is due to signalling from other regions in the brain, including the anterior hypothalamus (32, 42).

There are specialized neurons in the hypothalamus called osmoreceptors. These become active when the osmolarity of the extracellular fluid in the brain rises. Increased extracellular fluid osmolarity causes the osmoreceptor cells to shrink. When the osmoreceptor cells undergo shrinkage the rate of firing increases, promoting water retention and activating the thirst circuitry of the limbic system (32, 42).

Hypothalamic circuitry controls feeding behaviour. Signals from adipose tissue, the stomach and the pancreas either stimulate or inhibit food intake by acting on central targets in the hypothalamus. Neurons located in the lateral hypothalamic area are involved in activating the hunger drive. This is thought to occur via the release of orexin. Another area of the hypothalamus implicated in the regulation of feeding is the arcuate nucleus (Arc). Neurons in the Arc produce NPY, a 36-amino acid peptide. Animal

studies have shown that injections of exogenous NPY elicits a dose-dependent increase in feeding (32, 44). The ventromedial nucleus of the hypothalamus (VMH) is involved in satiety. The Arc also contains receptors for leptin. Leptin is a satiety factor that is produced by adipocytes. Leptin informs the brain about the amount of fat deposited in the body. It is proposed that leptin reduces food intake by decreasing the production of NPY in the Arc. Leptin also acts via α-MSH, which is a POMC (proopiomelanocortin) derived peptide, produced in the Arc. α-MSH inhibits feeding behaviour by acting on the melanocortin-4 receptor (MC4R). POMC is an anorexigenic peptide, that is proteolytically cleaved to produce a number of other peptides (γ-MSH, α-MSH, ACTH, β-MSH, and β-endorphin, in that order) (32, 44). Mutations in MC4R are the most common cause of monogenic obesity (45).

Humans are diurnal, indicating that they are more active, have a slightly higher body temperature, and secrete hormones that mobilize the body's energy stores during the day time. This 24 hour period is an evolutionary adaptation of the solar cycle of light and dark. The presence of a light-dark cycle establishes a circadian rhythm, but does not maintain it. The suprachiasmatic nucleus (SCN) in the hypothalamus is the master clock of the body. The SCN is located in the anterior hypothalamus, above the optic chiasm. This neuronal structure in involved in circadian rhythm generation and its regulation. The SCN receives input from specialized retinal ganglion cells (RGCs) that provide photic information that is used to synchronize the circadian rhythm with the natural light-dark cycle. In mammals, the circadian rhythm system requires three components: (1) photoreceptors and the visual pathways to mediate entrainment to light, (2) circadian

pacemaker cells (in the SCN), and (3) efferent projections that couple the pacemakers to the neural systems that express the circadian cycles. An alteration in any of these components will result in a dysfunctional circadian rhythm. The neurons in the SCN have an endogenous cellular rhythm. If SCN neurons are removed from the brain and placed in culture, the cells retain their circadian rhythm of action potential activity, neuropeptide secretion, and metabolic activity. The endogenous nature of the SCN neurons is also evident when an animal is placed into complete darkness. The overall pattern of activity and feeding versus sleeping remains the same. The cycle tends to extend to 25 hours when in constant darkness, however, the main point is that these cells are able to retain the circadian rhythm in the absence of an external light cue (32, 43).

GENETIC CAUSES OF PWS

PWS is caused by a lack of expression of genes on the paternally derived chromosome 15q11-13. These genes are known to be imprinted; meaning that under normal circumstances, the alleles of the genes in this region are only expressed from one chromosome and not the other, depending on the parent of origin (10, 12). Approximately 70% of PWS cases are caused by a *de novo* deletion on the paternal chromosome 15q11-13. Maternal uniparental disomy (UPD) accounts for ~25% of PWS cases. When this occurs, the individual has both chromosome 15's from maternal origin, and no chromosome 15 from paternal origin. The remainder of cases are due to genomic imprinting defects (microdeletions or epimutations), or a chromosomal translocation involving the paternally derived chromosome 15 (see Figure 1.2) (10, 12). Of the

deletion genotypes, there are also two distinct subtypes. There is a type I deletion which is 500 kb longer than the type II deletion. These two different deletion sizes arise because of two different breakpoints centromeric to the PWS critical region. Of individuals with PWS resulting from a deletion, 40% have a type I deletion and 60% have a type II deletion. The PWS critical region has a syntenic region in mouse, which is also imprinted (10, 12). This is discussed further in the next section.

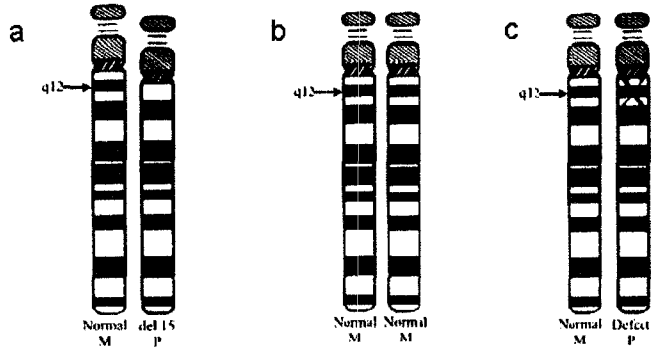
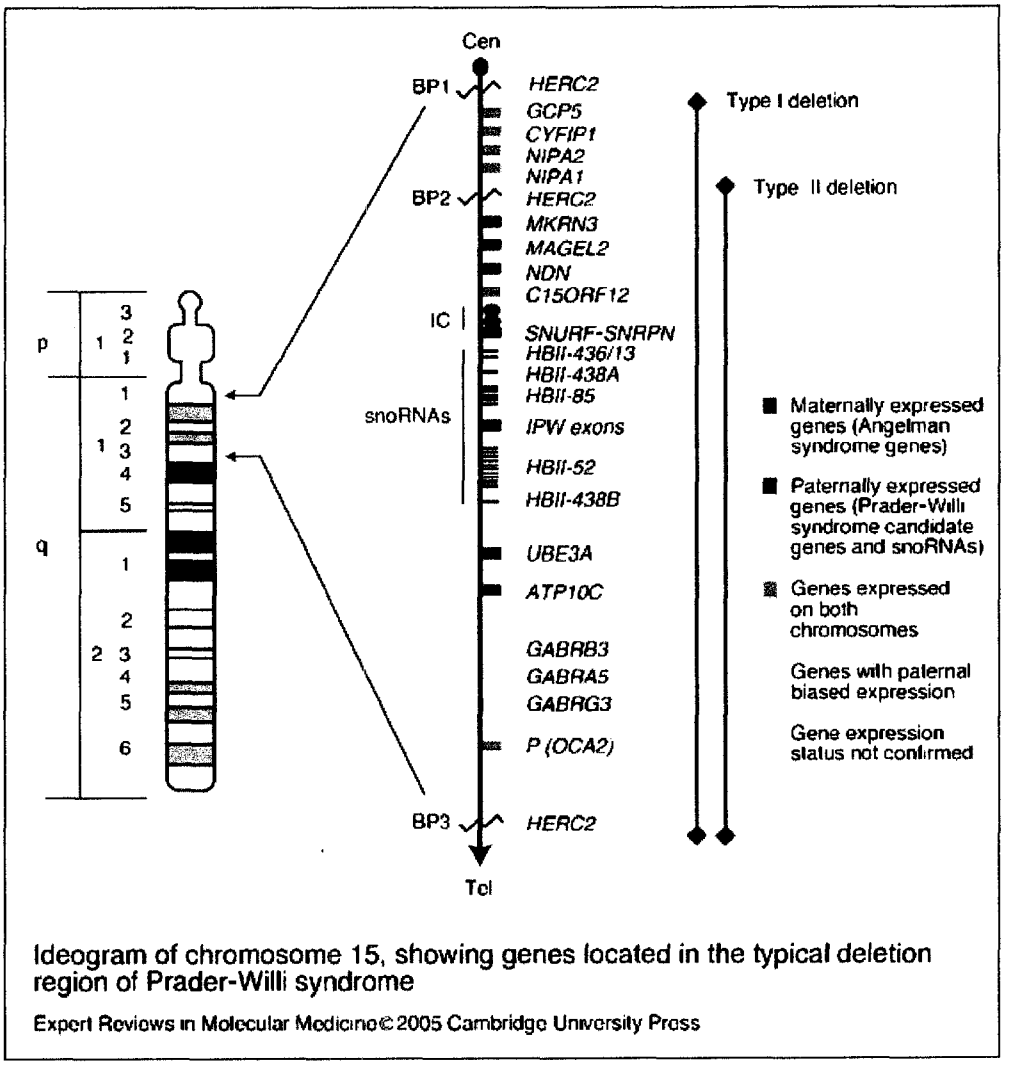


Figure 1.2 Chromosomal subtypes that cause PWS. M; maternal. P; paternal. (a) 70% of PWS cases are due to a deletion on the paternal chromosome 15, which is represented here. (b) 25% of PWS cases are caused by UPD of the maternal chromosome 15, which is represented here. (c) The remaining cases of PWS are caused by defects in the imprinting center, which is represented here, or due to chromosomal rearrangements involving the paternal chromosome 15 (not represented here). Figure generated from data on PWSA website. http://www.pwsausa.org/index.html

Diagnostic testing for PWS can be quite complex. The available tests include fluorescent *in situ* hybridization (FISH), informative microsatellite polymorphism analysis for UPD15, and parent-specific methylation status using either methylation-sensitive enzymes and a Southern blot or methylation-sensitive oligonucleotide primers and PCR (polymerase chain reaction) (1, 6). 99% of PWS cases can be detected due to abnormal, maternal-only pattern methylation status at the SNRPN locus on chromosome 15q11-q13 (1).

GENES WITHIN THE PWS REGION; 15q11-q13

Contained within the chromosomal region 15q11-q13 are numerous genes and genetic elements. The area of imprinting extends between BP2 and *ATP10C* (refer to Figure 1.3). The area that is critical to the causation of PWS lies between BP2 and *IPW* (refer to Figure 1.3). Within the 2 Mb region that is imprinted, there are 4 paternally expressed, protein coding genes; *MKRN3, MAGEL2, NDN,* and *SNURF-SNRPN,* as well as snoRNAs transcribed from the region around the imprinting center (1, 6). These are important to note, since it is likely that the combined loss of expression of these genes leads to the PWS phenotype.



Ideogram of chromosome 15, showing genes located in the typical deletion region of Prader-Willi syndrome

Figure 1.3 The typical deletion region for PWS and the genes located within this region. BP1 and BP2 refer to the two breakpoints at the centromeric end of the region. BP3 will recombine with either BP1 or BP2 to create the two deletion subtypes. There are a number of genes within this region, however, not all of them are protein coding. For example the region telomeric to the imprinting center (IC) contains genes that are transcribed to snoRNAs (small nucleolar RNAs), but not translated. The genes that are expressed exclusively from the maternal chromosome are shown in red, and those genes expressed exclusively from the paternal chromosome are shown in dark blue. Biallelically expressed genes are shown in green. Figure from **Bittel DC, Butler MG.** Prader-Willi syndrome: clinical genetics, cytogenetics and molecular biology. *Expert Rev Mol Med.* 2005;7(14):1-20.

Both *MAGEL2* and *NDN* (necdin) are imprinted genes located in the PWS critical region on chromosome 15. Also, both of these genes belong to the same family of genes; the MAGE family. The term MAGE comes from the identification of some family members in cancers; melanoma-(associated) antigen (46-48). There are two subgroups of *MAGE* genes; (I) *MAGE-A, -B*, and *-C* genes, which are generally only expressed in tumour or germ cells, and (II) consisting of the remaining *MAGE* genes, not represented in *-A, -B,* or *-C* subfamilies. The *MAGE* genes in subgroup II are expressed in various normal adult human tissues (46-48). The common thread among all the *MAGE* genes is the presence of a MAGE-homology domain (MHD). The MHD produces a protein structure that consists of 4 α-helices and 5 β-sheets (48). Figure 1.4 outlines the entire MAGE family, including the subgroups and shows the hallmark MHD in each member.

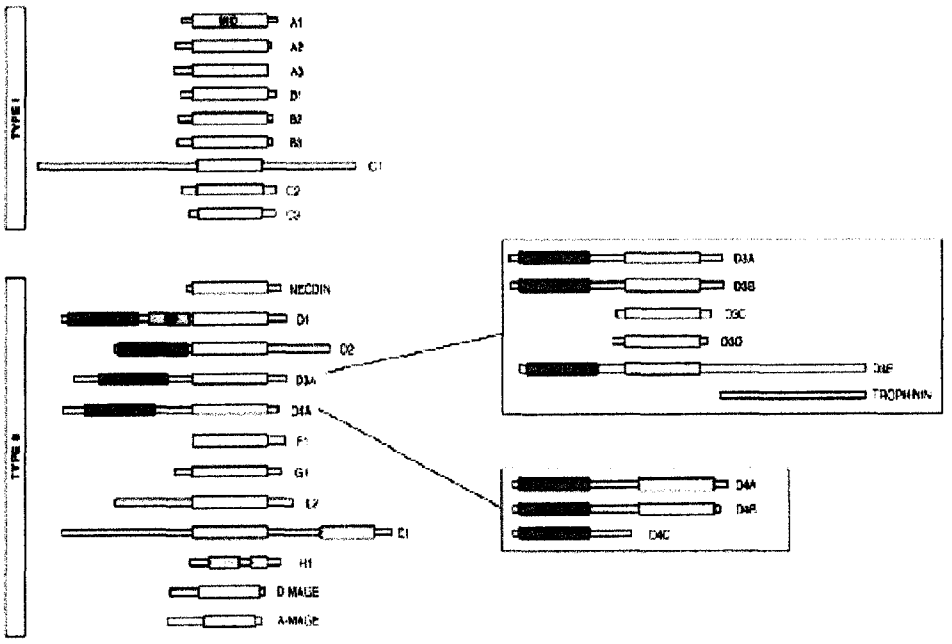


Figure 1.4 The MAGE family of genes. The MAGE genes are shown here separated by subgroup. The MHD is shown in yellow and the MHD2 is shown in green. The IRD (interspersed repeat domain) is shown in orange. The MHD2 is a second region that is loosely conserved in some of the MAGE genes. The IRD consists of 25 tandem repeats of WQXPXX. This hexapeptide repeat is not found in any other protein, but is well conserved between MAGE-D1 in mouse, rat, and human. The two genes that are of most interest are necdin and MAGE-L2, as they are genes involved in PWS. This figure is from **Barker PA, Salehi A.** The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. *J Neurosci Res.* 2002;67(6):705-12.

Necdin (*NDN*) is the best characterized MAGE family member. Overexpression of *NDN* can induce cell cycle arrest, suggesting its involvement in cell cycle arrest and maintenance of cell stability (46). This is especially true for neurons where *NDN* is implicated in neuronal terminal differentiation (2). *NDN* is also important in axonal outgrowth and fasciculation, as evidenced by defects in these processes in neurons derived from *Ndn*-null mice (2). *MAGEL2* is a single exon gene, with 51% homology to *NDN*, and its function is currently unknown. It is assumed that *MAGEL2* will have a

similar role to that of *NDN*. *NDN* is expressed in a much wider range of tissues, whereas *MAGEL2* is most strongly expressed in the hypothalamus. Therefore, defects in neuronal/axonal outgrowth and fasciculation in hypothalamic neurons resulting from a lack of *MAGEL2* expression is a logical explanation for the phenotype seen in PWS.

Magel2 KNOCK-OUT MICE

The chromosomal region 15q11-q13 involved in PWS has a region of conserved synteny on mouse chromosome 7C (Figure 1.5). This syntenic region allows for relative ease in generating mouse models for PWS. These mouse models include a UPD model, with a maternal duplication of 7C, mice with a deletion on 7C that is comparable to the deletion seen in PWS, as well as knock-out models for individual genes.

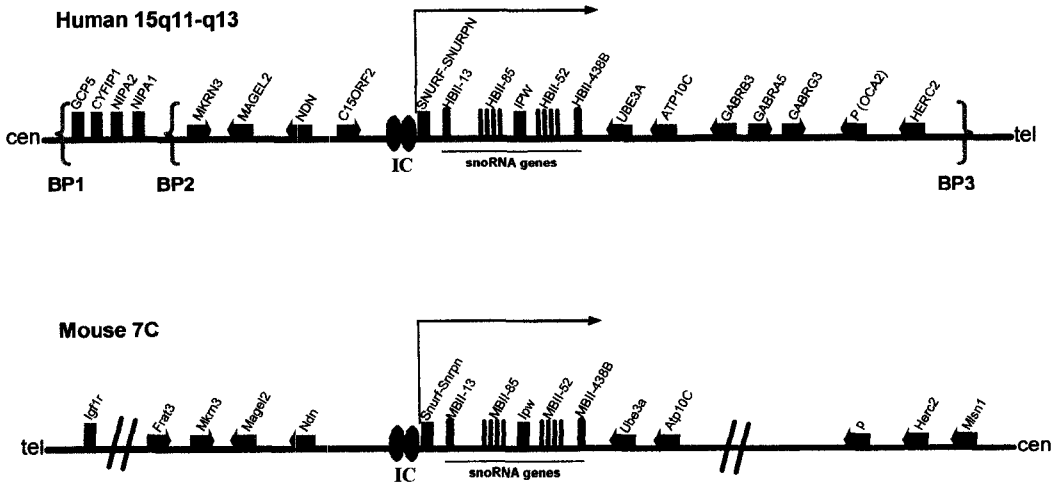


Figure 1.5 Human chromosome 15q11-q13 and the syntenic region in mouse (7C). The similarities between the human region commonly deleted in PWS and mouse chromosome 7C are quite distinct. The mouse chromosomal region is also under control of imprinting. There is another gene in the region of Magel2 and Ndn in the mouse; Frat3. Blue arrows and boxes indicate paternal expression. Red indicates maternal expression. Green indicates either biallelic expression or imprinting status undetermined. This figure was generated from several sources; see references (6, 12, 49).

A collaborating laboratory (C. L. Stewart, Frederick, MD) generated a mouse strain that is deficient for *Magel2*. The targeting of the *Magel2* locus was performed in W9.5 (129S1) embryonic stem cells. The bacterial artificial chromosome (BAC) clone

RPCI24-232N8 was used to construct the targeting vector, designed to eliminate the open reading frame (ORF) of *Magel2* by replacing it with an in frame LacZ expression cassette. The LacZ expression cassette then allows for identification of cells transcribing from the *Magel2* promoter. Restriction length fragment polymorphism (RFLP) analysis was used to detect the wild-type and null alleles. A correctly targeted clone was used to establish a colony of *Magel2* deficient mice, which were subsequently backcrossed onto a C57Bl/6 genetic background. The targeting strategy is shown in Figure 1.6.

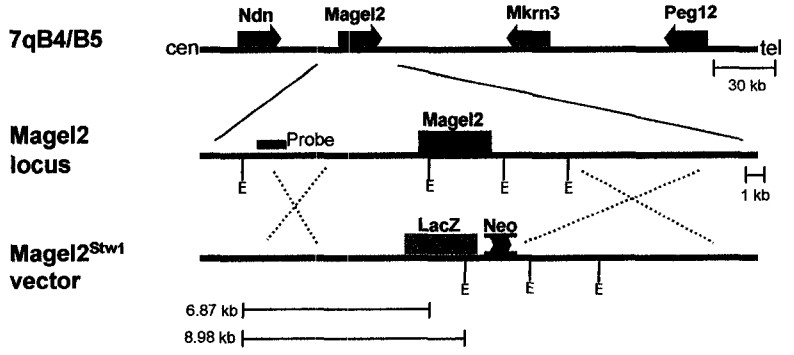


Figure 1.6 Targeting strategy and generation of the *Magel2*-null mouse. 7qB4/B5 is the mouse locus syntenic with PWS locus at chromosome 15q11-q13 in humans. Magel2^{Stw1} vector is the LacZ knock-in targeting vector containing a Neo minigene flanked by loxP sites (small arrows on Neo gene). The dotted lines represent the homologous recombination event. The EcoRI restriction sites are shown with the letter E. The probe is represented in purple. The un-targeted chromosome and the targeted chromosome will produce different fragments when digested with EcoRI and probed with the probe indicated. The targeted chromosome will produce a fragment of 8.98 kb, while the un-targeted chromosome will produce a fragment of 6.87 kb.

In the adult brain, *Magel2* expression was most prominent in the paraventricular nucleus (PVN), the suprachiasmatic nucleus (SCN), and the supraoptic nucleus (SON) of the hypothalamus. *Magel2* is also expressed in other regions of the hypothalamus, including the arcuate nucleus (Arc) and the ventromedial hypothalamus (VMH) (3). Figure 1.7 shows *Magel2* expression in an adult mouse brain.

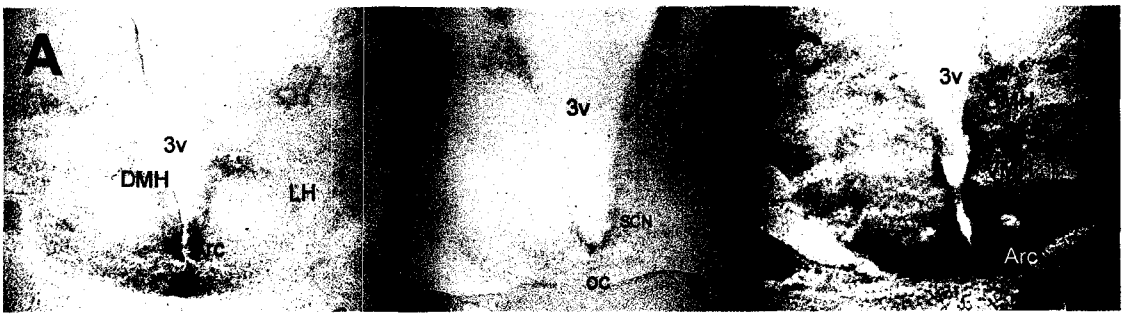


Figure 1.7 *Magel2* expression in the hypothalamus. LacZ reporter gene (blue) under the *Magel2* promoter in the *Magel2*-null mice. (A) E14.5, (B) E16.5, and (C) E16.5, all cryosectioned in the coronal plane at 60mm. Images taken at 10X. 3v = third ventricle, Arc = arcuate nucleus, DMH = dorsomedial hypothalamus, LH = lateral hypothalamus, oc = optic chiasm, SCN = suprachiasmatic nucleus, VMH = ventromedial hypothalamus.

Magel2 expression, in the adult central nervous system (CNS), is rhythmic in a light-dark (LD) cycle, with expression peaking in the late day. There is a 3.7-fold difference in expression between peak and trough in the SCN, 5.1-fold difference in the Arc, and a 10.3-fold difference in the VMH. When mice are subjected to constant darkness (DD), the rhythmic expression of *Magel2* persists (Kozlov *et al*, unpublished data). *Magel2*-null mice were generated to investigate the role of Magel2 in circadian rhythm, and see the effects of a lack of Magel2.

The *Magel2*-null mice survive to adulthood. By recording running wheel activity, circadian behaviour was analyzed in wild-type and *Magel2*-null littermates. Under LD conditions, the null mice shown a relatively normal pattern of activity, however, the null mice ran significantly less than their wild-type littermates (Figure 1.8). Also, the null mice ran in more frequent, but shorter bouts than their wild-type littermates, as evidenced by the lack of consolidation on the *Magel2*-null actogram. When released into constant darkness (DD), the *Magel2*-null mice exhibit a severe lack of consolidation in their

pattern of running activity, whereas the wild-type mice show preservation of their running activity as seen in LD conditions (Figure 1.8).

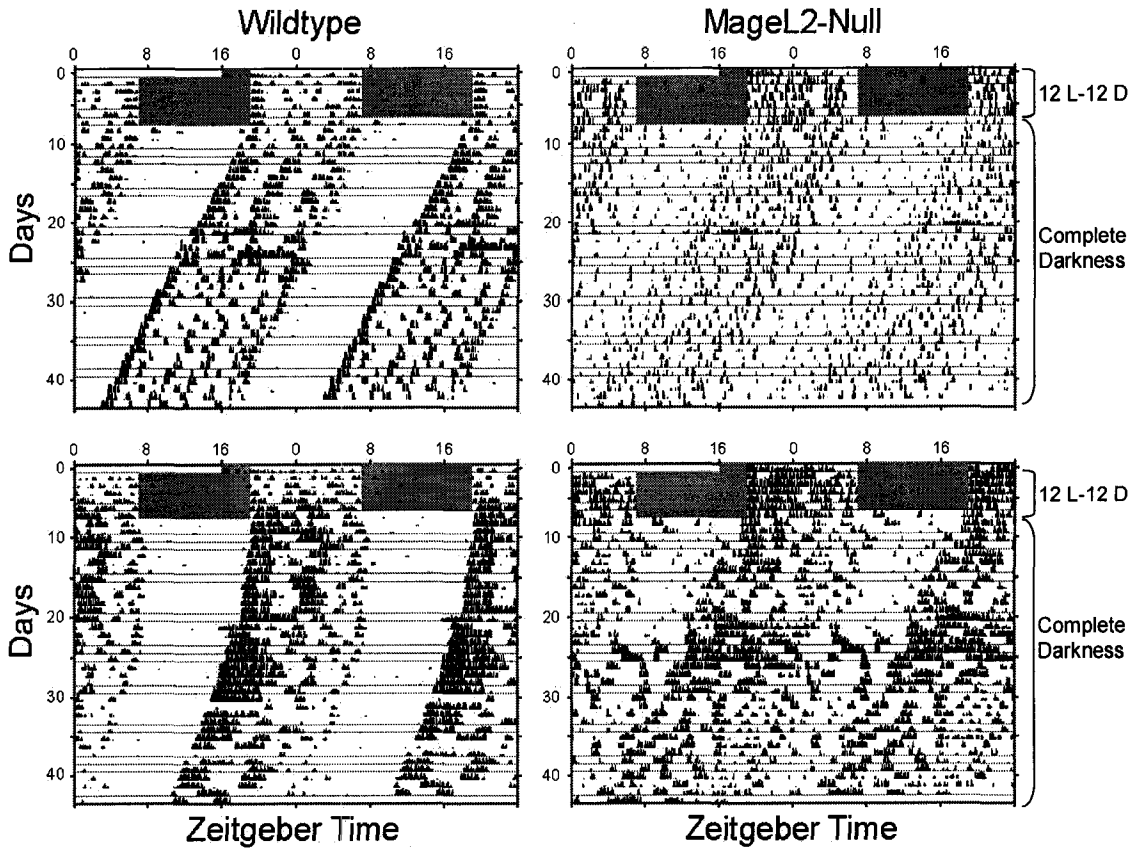


Figure 1.8 Actogram results of two wild type mice and two *Magel2*-null mice in 12 hours light and 12 hours dark (12 L-12 D), and in complete darkness. Note that the *Magel2*-null mice exhibit less activity during 12 L-12 D, and a lack of consolidated activity during complete darkness (Kozlov *et al*, unpublished data). Zeitgeber time is a standard of time based on the period of a zeitgeber. A zeitgeber is a synchronizing agent capable of resetting a pacemaker or synchronizing a self-sustaining oscillation. A zeitgeber hour is the unit of time corresponding to 1/24 of the period of a zeitgeber (50).

HYPOTHESIS

The rationale behind the experiments and results in this thesis are based on two hypotheses. First, there is a lack of *MAGEL2* expression in PWS. Hyperphagia and obesity are major components of PWS. Therefore, my hypothesis is that single gene

mutations in *MAGEL2* could also cause hyperphagia and obesity in non-PWS individuals. Second, *Magel2* is expressed most highly in the hypothalamus. Lack of expression of *Magel2* in mice will elucidate the normal role of Magel2. My hypothesis is that *Magel2*-null mice will show hypothalamic symptoms, such as hyperphagia/obesity and reproductive abnormalities, as well as dysfunction of the circadian rhythm system (discussed earlier in Chapter 1). I have used the *Magel2*-null mice to examine the effect of a lack of *Magel2* in obesity and fertility.

CHAPTER 2 ★ MUTATION ANALYSIS OF DNA FROM OBESE AND HYPERPHAGIC INDIVIDUALS

The results reported in this section are published: *Evaluation of Prader-Willi Syndrome Gene MAGEL2 in Severe Childhood-Onset Obesity.* **M. A. O'Neill**, I. S. Farooqi, & R. Wevrick. Obesity Research, Vol. 13 No. 10, October 10th, 2005.

INTRODUCTION

In the murine brain, *Magel2* is highly expressed in the hypothalamus, specifically in the suprachiasmatic nucleus (SCN), the supraoptic nucleus (SON) and the arcuate nucleus (Arc) (3). The Arc is involved in the regulation of feeding behaviour. *MAGEL2* is one of four genes that are not expressed in PWS (3, 6, 8). Obesity and hyperphagia are hallmarks of PWS, thought to be due to hypothalamic dysfunction. As *MAGEL2* is expressed in the area of the hypothalamus implicated in the regulation of food intake, it seems likely that lack of expression of *MAGEL2* in PWS contributes to the hyperphagia and obesity seen in PWS. From this idea, it can then be proposed that mutations that affect *MAGEL2* only (and not the other 3 genes affected in PWS) *could* result in childhood-onset hyperphagia and obesity, without the other traits of PWS. Therefore, we hypothesized that alterations in *MAGEL2* may result in non-syndromic obesity. To test this hypothesis, we screened DNA from children with non-syndromic (not resulting from any known cause) obesity, defined as (1) severe obesity before the age of 10 and (2) with symptoms of hyperphagia, for mutations in the *MAGEL2* gene.

MATERIALS AND METHODS

SUBJECT DNA

Farooqi *et al* calculated the BMI for each subject, with respect to sex and age, and determined all subjects to be in the severely obese centile for their respective sex and age peer group (45). These subjects were originally part of a cohort of 500 that do not carry mutations in the *MC4R* gene, the gene responsible for the most common form of

monogenic obesity. The subjects used in this analysis are defined to be hyperphagic, which is of interest as a major characteristic of PWS is hyperphagia.

PCR

Each primer set was run on the following program of cycling temperatures: (1) 95°C, 5:00 (2) 95 °C, 0:30 (3) T_M °C, 0:30 (4) 72 °C, 0:30 (5) GOTO (2), REPEAT 29X (6) 72 °C, 10:00 (7) 95 °C, 5:00 (8) RAMP 0.1 °C/sec TO 20 °C (9) 4 °C, FOREVER (10) END Each reaction had a final PCR Reaction Buffer concentration of 1X (10X is 200 mM Tris pH 8.4, 500 mM KCl. Invitrogen, Part # Y02028), final dNTPs concentration of 0.2mM (Invitrogen, Part # 55082(dATP), 55083 (dCTP), 55084 (dGTP), 55085 (dTTP)), and a final concentration of Taq Polymerase 0.025U (Invitrogen, Cat. # 10342-020). The final concentration of $MgCl_2$ (Invitrogen, Part # Y02016) was 2mM for Fragments 1, 3, 4, & 5, and 1.5mM for Fragment 2.

Fragment #	Forward Primer	Reverse Primer	$[MgCl_2]_f$	T_M	PCR Product Size
1	CCG CTA AAT CAT TGA TGA CTC C	GAC CTC CCA GTC ACT CAG ATT TAG	2mM	55°C	652bp
2	CCA CCT TCC TGA TGG CTA CAG	CCC GGA GAG ACA CTT GCG ACC TCA G	1.5mM	54°C	451bp
3	CAG GGC CCT GGG TCT CTC TGA AAG CCC	TAC TCT CGG AGG ATG ACT TT	2mM	57°C	233bp
4	TGC CTG TCC AGC GCT CG	ATT CGC CTG TAC TCT A	2mM	55°C	399bp
5	GAA ATA CTA AGA AGC TCA TC	GGA GCG AGA TCT CTG CTA CAC C	2mM	55°C	322bp

Table 2.1 Primer sequences, final $MgCl_2$ concentration, T_M of reaction, and size of PCR product for each of the *MAGEL2* overlapping fragments.

PRIMER DESIGN & GENOMIC PCR

We designed primers to cover the entire *MAGEL2* open reading frame (ORF) in five overlapping PCR products (Figure 2.1). The primer pairs were designed to have similar T_Ms, and to avoid any primer dimer formations (Table 1). The sequence of each PCR

product also had to have a relatively uniform melting temperature across the entire strand, as this is necessary for analysis using the Transgenomic WAVE™ DNA Fragment Analysis System.

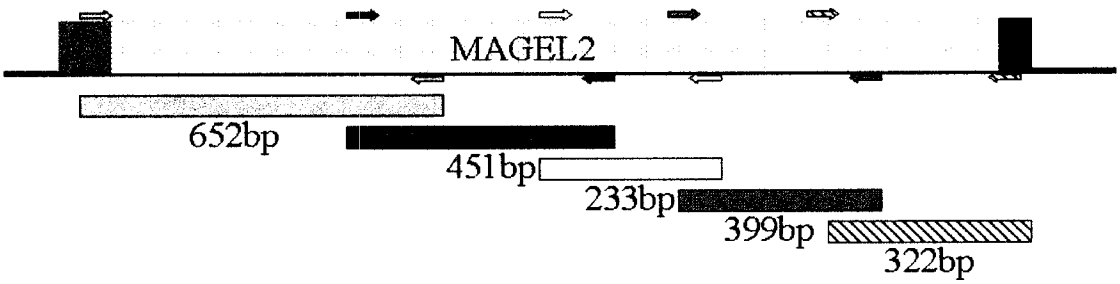


Figure 2.1 *MAGEL2* gene schematic showing the overlapping primer pairs (arrows), and their respective PCR products.

MUTATION ANALYSIS

We used the WAVEMAKER Software 4.1 (Transgenomic, Part # 672001) to analyze the input sequence for each PCR product, which determined the specific conditions for each. These conditions included oven temperature and the continuous flow variations in the concentrations of buffers A, B, C, and D (Table 2.2). These buffers differ in their respective concentrations of acetonitrile and triethylammonium acetate (TEAA). Buffer A: 0.1M TEAA solution, pH 7.0. Buffer B: 0.1M TEAA containing 25% acetonitrile, pH 7.0. Wash Solution C: 8% acetonitrile, pH 7.0. Wash Solution D: 50% acetonitrile, pH 7.0.

PCR Fragment #	Oven Temperature
1	62.3°C
2	62.7°C
3	63.1°C
4	58.4°C
5	61.5°C

Table 2.2 The oven temperature used for each of the *MAGEL2* PCR products as analyzed using WAVEMAKER software.

SEQUENCING

The PCR reactions were gel extracted and purified (QIAGEN QIAquick® PCR Purification Kit, Cat. # 28106). Purified DNA was sequenced using Amersham's DYEnamic ET Terminator Cycle Sequencing Kit (Cat. # US81050), with each respective primer (see Table 2.1) and read on an Applied Biosystems 377 XL Sequencer.

RESULTS

We examined the single exon *MAGEL2* gene for sequence alterations in hyperphagic individuals with non-syndromic obesity. We amplified five DNA fragments with overlapping coverage of the 1589 nucleotide open reading frame and tested the fragments for sequence variation by denaturing HPLC (D-WAVE). Of the probands screened, 27 of 96 gave D-WAVE patterns suggesting heterozygosity, and these samples had sequence alterations. Twenty five probands had a single nucleotide change, a G to C transversion, which results in a V272L polymorphism (Figure 2.2).

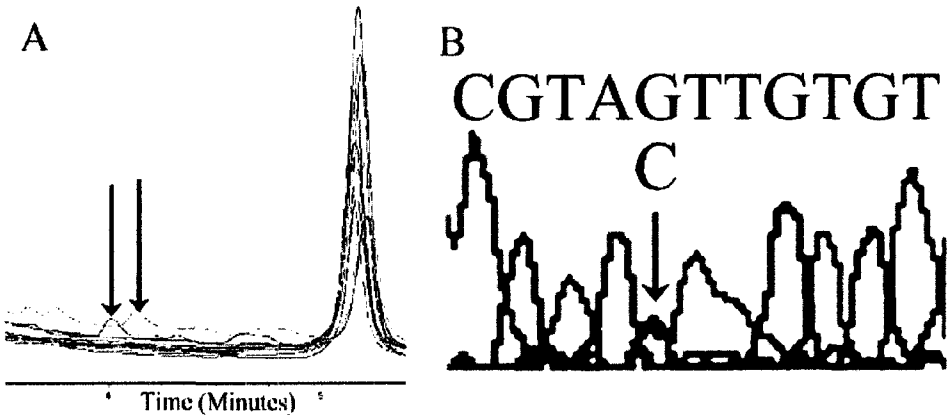


Figure 2.2 (A) DHPLC analysis results showing 2 of the 25 proband samples with a heteroduplex peak (arrows) and homoduplex peak (large peak to the right). (B) The sequencing results show a G to C transversion, resulting in a Valine to Leucine polymorphism at codon 272.

The other two probands had a single nucleotide change, a C to A transversion, with results in a L331I polymorphism (Figure 2.3).

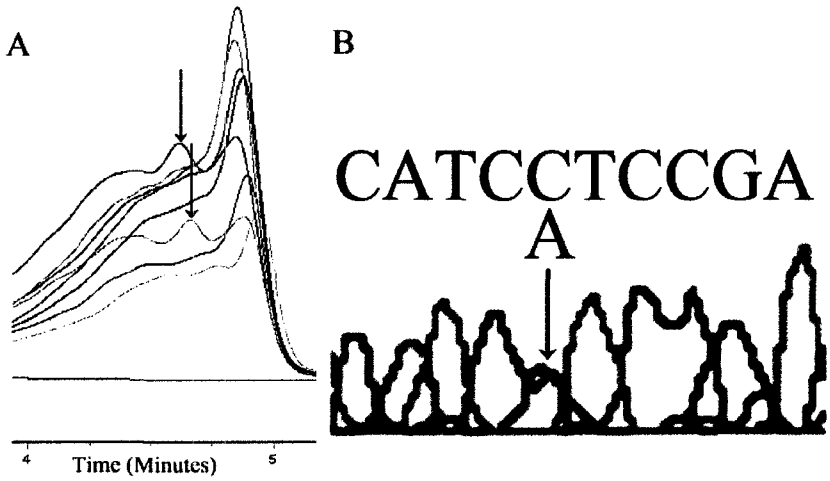


Figure 2.3 (A) DHPLC analysis results showing two probands with a heterduplex peak (arrows) and homoduplex peak (large peak to the right). (B) The sequencing results show a C to A transversion, resulting in a Leucine to Isoleucine polymorphism at codon 331.

All probands with nucleotide changes are heterozygous for their respective nucleotide change. Furthermore, in the ten control samples that were used to optimize the PCR

conditions and HPLC mutation analysis protocols, we found two nucleotide differences. The first is a T to C transition at codon 284, which results in a S284S polymorphism (Figure 2.4). The second nucleotide change is the same C to A transversion at codon 331 that was also found in two probands, resulting in a L331I polymorphism.

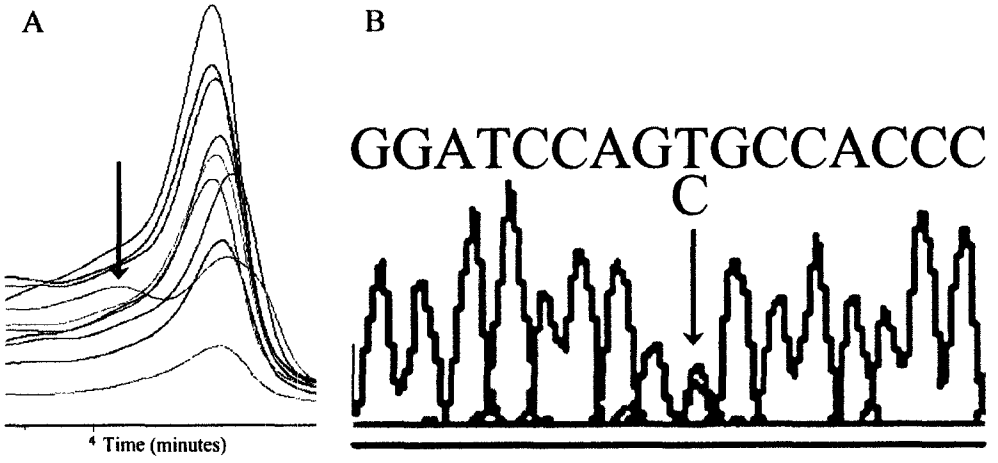


Figure 2.4 (A) DHPLC analysis results showing a control sample with a heteroduplex peak (arrow) and homoduplex peak (large peak to the right). (B) The sequencing results show a T to C transition, resulting in a Serine to Serine polymorphism at codon 284.

To determine if the high frequency of the V272L change was specific to the severe childhood-onset obesity population, we also screened additional control samples for this nucleotide change. We found that the V272L polymorphism occurred with approximately the same frequency in each population (28% in proband samples, 30% in control samples). Of the polymorphisms reported here, only one lies within the MAGE homology domain (L331I). This amino acid change from Leucine to Isoleucine actually makes the protein more homologous with the mouse and rat MAGEL2 proteins (Figure 2.5 B). Based on this improved homology, we concluded that this amino acid was a polymorphism rather than a mutation.

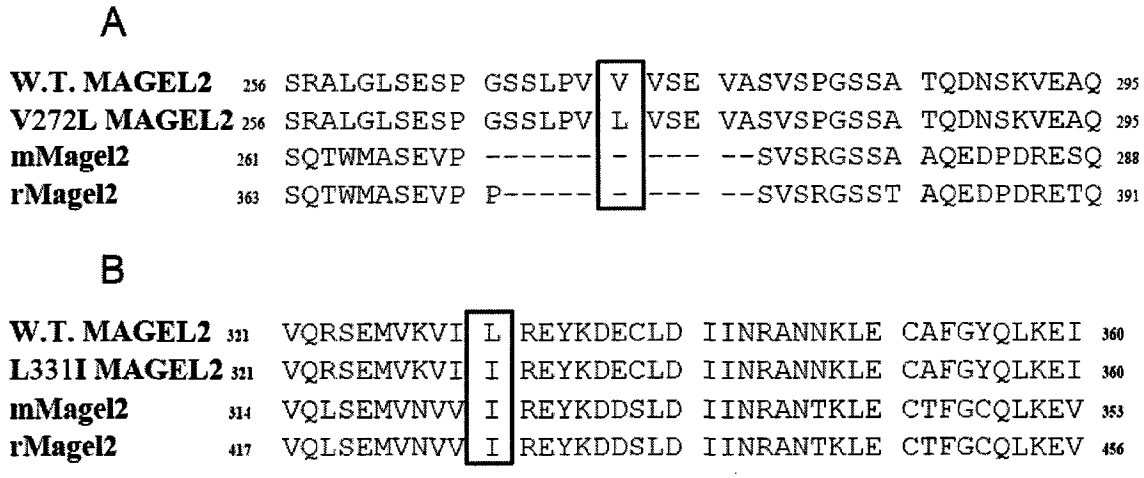


Figure 2.5 Amino acid alignment of human, mouse, and rat MAGEL2 proteins. (A) Wild-type MAGEL2 (W.T. MAGEL2), valine to leucine polymorphic allele (V272L MAGEL2), mouse Magel2 (mMagel2), and rat Magel2 (rMagel2). (B) Wild-type MAGEL2 (W.T. MAGEL2), leucine to isoleucine polymorphic allele (L331I MAGEL2), mouse Magel2 (mMagel2), and rat Magel2 (rMagel2).

For the other two polymorphisms we reported (V272L, S284S), we used amino acid similarity, as well as control population frequency to conclude that these were very likely polymorphisms rather than mutations. The V272L polymorphism occurs in an 11 amino acid stretch that is unique to the human MAGEL2 protein, and does not occur in either the mouse or the rat Magel2 proteins (Figure 2.5 A).

DISCUSSION

Our *MAGEL2* screening results in severely obese children indicate that *MAGEL2* is not commonly mutated in probands with severe childhood obesity. If *MAGEL2* were directly involved with hypothalamic neural circuits that regulate appetite, we would have expected alterations in the *MAGEL2* ORF to be present in the screened samples of unexplained childhood obesity. Any mutation that is present on the maternal allele is

very likely negligible, as the maternal allele of *MAGEL2* is silenced. In contrast, if a mutation was paternally inherited, the phenotypic consequences could be much more profound, as this is the only actively expressed allele. In additional screens for the role of PWS gene in non-syndromic obesity, it is important to determine parental origin of the mutant allele. Any major nucleotide change found in the screened individuals, for example a nonsense mutation, would need further investigation as to the parent of origin of that specific allele in order to determine the phenotypic consequences of the mutation.

Oeffner *et al* conducted a similar screening study of a PWS gene (*NDN*) for sequence aberrations (51). Their study included a sample population of severely obese children and adolescents, as well as a sample population of healthy underweight probands (51). Their conclusions are similar to ours in that the polymorphisms that were identified were not significant, and had approximately the same allelic frequencies in both the obese and underweight sample sets (51).

It is doubtful that our sample size was too small since analogous studies of screening a candidate gene for its involvement in childhood obesity have used similar sample sizes (51, 52). However, it is possible that the criteria for severe childhood obesity was too stringent, and should have included at least one other characteristic of PWS. For example, *MAGEL2* mutations may be more syndromic, and be present in severely obese children with mental retardation, which would have been excluded in this study. In this study, children with non-syndromic obesity, defined as (1) severe obesity before the age

of 10 and (2) with symptoms of hyperphagia, did not test positive for any mutations in the *MAGEL2* ORF.

CHAPTER 3 ★ WEIGHT GAIN AND FERTILITY IN *Magel2*-NULL MICE VERSUS WILD TYPE MICE

INTRODUCTION

Obesity is the cardinal feature of PWS. The obesity is accompanied by and due to hyperphagia, which usually starts in early childhood (6, 10). Hypogonadism and a significant reduction in the level of sex hormones are also features of PWS. The majority of individuals with PWS are infertile (5, 6, 8-10, 12, 15). PWS is a genetic syndrome caused by the loss of expression of four protein-coding genes; *MKRN3*, *MAGEL2*, *NDN*, and *SNURF-SNRPN* (1, 6). It is known that MAGEL2 is expressed strongly in the hypothalamus (53). However, the role of *MAGEL2* in development and maintenance of the brain, specifically the hypothalamus, is still unknown.

To investigate the effect of a loss of *Magel2* expression on obesity, dietary and weight measuring experiments were conducted using *Magel2*-null mice. The mice were given one of three diets. The mice were given either a conventional diet, a diet used for breeding mice ("breeder diet"), or a diet with a high fat content ("high fat diet"). The mice were weaned to one food type and weighed weekly from the time of weaning to 12 weeks of age. The genotype of each mouse was determined from the ear notch biopsies that are done to number the mouse.

To determine the fertility of *Magel2*-null mice, an experiment was conducted and repeated. This experiment put either a wild type male mouse or a *Magel2*-null male mouse in a cage with 3 virgin wild type females for one week. After 1 week, all four mice were put into separate cages. The females were monitored for the appearance of pregnancy, as well as for the birth of any litters. This experiment is a simple way of

determining if there are any differences in the ability to reproduce between the wild type mice and the *Magel2*-null mice.

By performing a necropsy on sacrificed animals of both genotypes, I was able to measure and analyze any differences in organ weight and the weights of various sources of fat. The organs were weighed to determine if organ weight could account for any weight differences in the wild type mice versus the *Magel2*-null mice. Also, as a follow-up to the breeding experiments, the testes and gonadal tissue from each mouse were analyzed for any abnormalities in the reproductive system. Terminal bleeds were done at the time of necropsy to measure serum testosterone levels.

MATERIALS AND METHODS

GENERATION OF Magel2-NULL MICE

A collaborating laboratory (C. L. Stewart, Frederick, MD) generated a mouse strain that is deficient for *Magel2*. The *Magel2* locus was targeted using standard procedures in W9.5 (129S1) embryonic stem cells. The BAC (bacterial artificial chromosome) clone RPCI24-232N8 was used to construct a targeting vector. This targeting vector is designed to eliminate the *Magel2* ORF (open reading frame) and replace it with an in frame LacZ expression cassette. This expression cassette allows cells transcribing and expressing the *Magel2* locus to be identified because they now express the LacZ gene product. Once a correctly targeted clone was verified, it was used to establish a colony of Magel2-deficient mice using standard techniques that were backcrossed onto a C57BL/6

genetic background. Detailed information and a figure of the *Magel2*-null mouse generation can be found in Chapter 1 (Figure 1.6).

DIETARY COMPOSITION

The conventional and breeder diet are standard diets supplied by HSLAS (Health Sciences Laboratory Animal Services, University of Alberta). The conventional diet has 12.0% energy derived from fat. The breeder diet has 21.7% energy from fat. The higher energy from fat content in the breeder diet is due to its purpose in nourishing breeding females. The 60% energy from fat diet is from TestDiet (Basal purified diet with 60% energy from fat, blue; product # 58G9). Due to the higher fat content of this diet, the storage conditions are critical. The food must be stored at 2-4°C short term and at -20°C long term. This particular diet is highly perishable, and therefore required fresh food every day.

WEIGHT MEASUREMENTS

A scale with the accuracy of one decimal place was set up with a large plastic or glass beaker (≥1L), which was tared to read zero. Each mouse was picked up by its tail and placed into the beaker. The measurement was read, and recorded for the appropriate ear notch number. This was done on a weekly basis.

POST MORTEM PATHOLOGY

The mice used were M203 (W.T.), M204 (W.T.), M205 (*Magel2*-null), and M214 (*Magel2*-null). M203, M204, and M205 were 5 months and 1 day old (152 days), and

M214 was 5 months and 2 days old (153 days) on the day of euthanasia and necropsy. Post mortem pathology was done with Dr. Nick Nation of Health Sciences Laboratory Animal Services (HSLAS). For each mouse, Dr. Nation euthanized the mouse, did a terminal bleed, dissected out the organs, and took the testes and gonadal tissue. I collected the blood from the terminal bleed to test the testosterone levels (protocol below). Immediately after the terminal bleed, Dr. Nation took out the brain which was then flash frozen on dry ice. The heart, liver, and kidneys were dissected out next, and also flash frozen on dry ice. I collected the organs and fat for weight analysis. Dr. Nation formaldehyde fixed the testes and all gonadal tissue (i.e. prostate, scent glands, etc) for pathology and histology to look for any abnormalities. The testicular fat and peri-renal fat were also dissected out and flash frozen on dry ice, for weight analysis. The entire skin with subcutaneous fat was collected from M203 and M205.

Terminal Bleed

A 21-23g needle with a 5mL syringe was used. The needle was put in at the midline under the sternum at a 30° angle. A negative pressure was created by pulling back on the syringe plunger. The needle was moved around until there was flash of blood. From here, the plunger was pulled back to get the majority of the blood to flow into the syringe. An adult mouse should yield ~0.25-1.00 mL of blood. A volume of approximately 0.75 mL is considered good. The blood from the syringe was put into a labelled tube, and put on ice. The vials of blood were centrifuged at 4°C, at 4000 rpm for 20 minutes. There was a clot of cells at the bottom of the centrifuged tube. The serum/plasma above the clot of cells was moved to a fresh tube. The serum was frozen at -20°C. The serum was

shipped to a testing facility where it was tested for a serum level of testosterone. This is done using an antibody, and quantification of the signal.

ANATOMICAL WEIGHT MEASUREMENTS

The tissues weighed were brain, kidney, heart, liver, testicular fat pads, perirenal fat, and subcutaneous fat (for M203 and M205 only). The weight analysis was done on a scale sensitive to three decimal places. The scale was tared with an empty tube first, and then the tissue in its tube was weighed to obtain the weight of the tissue. The tissues were weighed in a frozen state.

EXPERIMENTAL MATING

Each male, approximately 15 weeks old, was placed into a cage containing three virgin females. The placement was for one week. A period of one week was used so that the male would encounter each female in estrous at least once during that week. After one week, the mice were all separated to individual cages. The females were monitored for pregnancy and subsequent birth of any litters. During the entire experiment, the mice were being fed a breeder diet to ensure healthy pregnancies and litters. The breeder diet is described above.

STATISTICAL ANALYSIS

Fisher Exact Probability Test (one-tailed)

The Fisher exact probability test is used for small sample numbers in the place of a χ^2 test (chi-squared test). In analysis of my results, I used the Fisher exact probability test to see

if being a Magel2-null male was significantly associated with the inability to impregnate females.

Fisher Exact Probability Test (one-tailed)

	Not pregnant	Yes Pregnant	
Magel2-null	a	b	a + b
W.T.	c	d	c + d
	a + c	b + d	N

$$p = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{N!\,a!\,b!\,c!\,d!}$$

T-Test

For each sample group (wf, mf, etc), descriptive statistics were obtained in Microsoft Excel. The ratio of the Standard Error's (SE's) between the two groups was compared (e.g. wf versus mf) dictated the type of t-test used. If the ratio of the SE's is less than two, a t-test assuming equal variance is used. If the ratio of the SE's is greater than two, a t-test assuming unequal variance is used. The t-tests were also calculated using Microsoft Excel.

RESULTS

Wild type mice and *Magel2*-null mice were fed different diet to determine if there is a difference in the weight gained. There were three different diets that the mice were fed; (1) conventional diet (12.0% energy from fat), (2) breeder diet (21.7% energy from fat), and (3) high fat diet (60.0% energy from fat). The mice were categorized by sex and

genotype. Therefore, there are four groups of mice per diet; (1) wild type female (wf), (2) Magel2-null female (mf), (3) wild type male (wm), and (4) Magel2-null male (mm). The mice were weighed weekly.

CONVENTIONAL DIET

Four litters were weaned and then fed the conventional diet (12.0% energy from fat). The breakdown of sex and genotype is as follows: wf = 8, mf = 8, wm = 6, and mm = 7. Table 3.1 shows the weight measurements over the weeks collected. Figure 3.1 shows the average of each group (e.g. wf) at each time point plotted against the other groups.

Table 3.1 Weight measurements of mice fed a conventional diet (12.0% energy from fat) from 4 weeks to 12 weeks of age. Shaded weights were collected closer to the 13th week rather than the 12th week, but are included here for convenience. Blank boxes indicate no weight measurement taken. The average of each group at each time point is at the bottom.

	Age (weeks)								
wf	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
M117			19.2		22.9	20.4	21.3	21.0	21.3
M119			20.7		24.2	22.2	23.3	22.8	24.9
M125			18.1		19.6	20.0	21.5	21.0	20.7
M126			19.2		20.4	20.3	20.8	20.7	21.4
M154		17.0	18.9	19.7	20.5	21.0	22.2	21.8	22.0
M156		14.9	17.6	17.5	18.3	18.9	18.0	19.3	19.7
M184	17.5	18.9		20.2	20.6	20.3	21.4	21.4	21.1
M185	16.8	18.7		20.3	21.7	21.7	23.2	23.1	24.1
mf									
M118			20.1		24.5	23.3	23.9	24.2	24.4
M124			18.5		20.9	21.1	21.4	21.0	21.1
M153		16.3	17.9	18.4	19.3	22.4	20.2	20.3	21.1
M155		16.2	17.1	17.3	17.3	17.2	17.7	18.3	18.2
M157		16.2	16.7	17.0	17.3	17.2	19.2	17.9	18.4
M183	15.8	17.0		17.7	17.9	18.9	20.3	21.3	21.9
M186	15.5	16.3		16.3	17.1	18.0	20.0	20.2	20.4
M187	16.1	18.4		18.7	19.9	20.2	22.1	22.2	23.0
wm									
M121			23.9		25.8	25.2	26.0	25.4	26.1
M123			24.3		25.4	24.2	25.5	25.5	25.8
M128			21.9		24.9	24.9	25.9	25.6	26.6
M129			22.3		24.6	24.5	24.9	24.9	25.4
M158		20.3	21.2	22.6	24.0	24.5	24.9	25.5	25.1
M189	19.7	21.7		23.7	24.6	25.6	26.7	26.3	27.1
mm									
M122			24.7		27.7	27.5	27.3	27.3	27.8
M130			24.3		27.1	27.4	28.0	27.8	29.4
M159		19.1	21.1	23.0	24.3	25.1	25.3	24.8	24.9
M160		19.4	21.3	22.5	23.3	22.6	21.9	22.5	23.6
M161		19.5	21.5	22.7	24.5	24.9	24.9	24.4	25.2
M188	18.7	21.2		21.6	22.1	22.9	24.6	24.1	25.1
M190	20.9	23.6		25.8	27.3	28.4	29.6	29.4	30.2

Average

	4	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
wf	17.2	17.4	19.0	19.4	21.0	20.6	21.5	21.4	21.9
mf	15.8	16.7	18.1	17.6	19.3	19.8	20.6	20.7	21.1
wm	19.7	21.0	22.7	23.2	24.9	24.8	25.7	25.5	26.0
mm	19.8	20.6	22.6	23.1	25.2	25.5	25.9	25.8	26.6

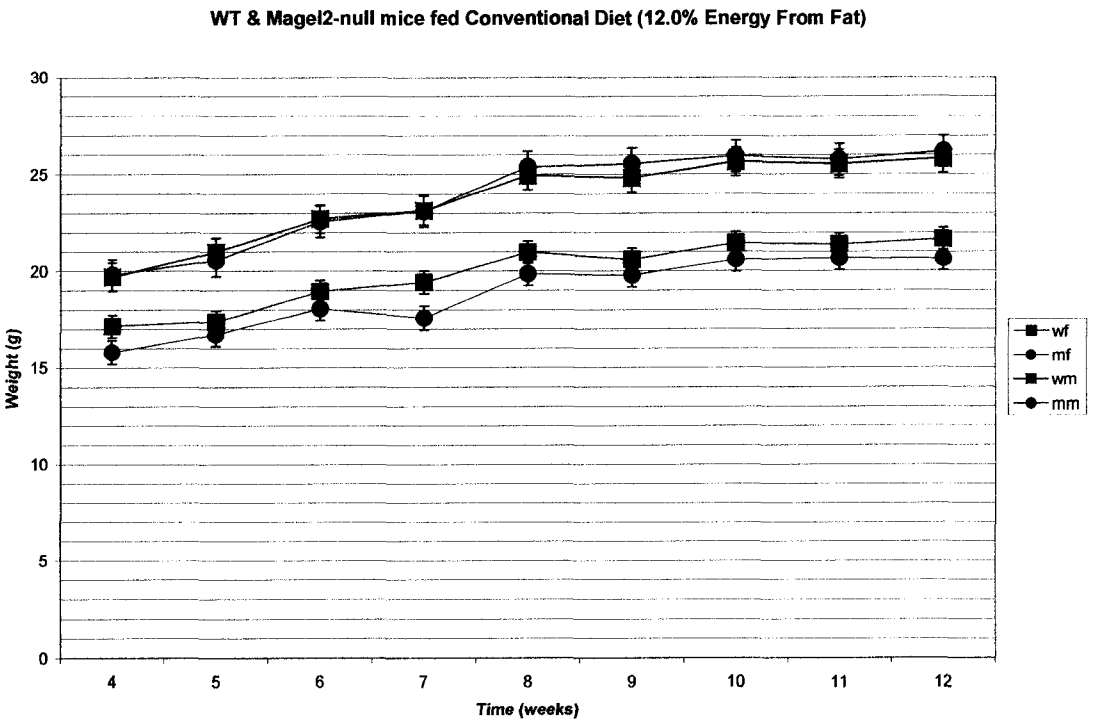


Figure 3.1 Wild type and *Magel2*-null mice fed a conventional diet. The data points are taken from the average weight for each group at each time point (see raw data at the bottom of Table 3.1). wf = wild type female, mf = *Magel2*-null female, wm = wild type male, mm = *Magel2*-null male. Error bars are SEM (standard error of the mean).

Statistical analysis results for differences between wild type and *Magel2*-null at 6, 9, and 12 weeks are shown in Table 3.2. There were no statistically significant differences between genotypes at any age. Full statistical analysis of each genotype and age comparison is detailed in Appendix A.

Table 3.2 T-test results for significant difference between wild type and *Magel2*-null mice fed a conventional diet at 6, 9, and 12 weeks. wf: wild type female, mf: *Magel2*-null female, wm: wild type male, mm: *Magel2*-null male.

	6	9	12
wf-mf	p=0.13	p=0.20	p=0.20
wm-mm	p=0.45	p=0.22	p=0.29

BREEDER DIET

Two litters were weaned and then fed a breeder diet (21.7% energy from fat). It is called a breeder's diet as it is fed to mice that are breeding (especially females) because it has a slightly higher fat content than the conventional diet. The breakdown of sex and genotype is as follows: wf = 2, mf = 3, wm = 3, and mm = 7. Table 3.2 shows the weight measurements over the weeks collected. Figure 3.2 shows the average of each group (e.g. wf) at each time point plotted against the other groups.

Table 3.3 Weight measurements of mice fed a breeder diet (21.7% energy from fat) from 4 weeks to 12 weeks of age. The average of each group at each time point is at the bottom. No data was recorded for week 7.

	Age (weeks)						
wf	**5**	**6**	**8**	**9**	**10**	**11**	**12**
M207	17.9	18.4	19.7	20.3	20.7	21.1	21.7
M210	17.1	18.6	19.3	19.8	20.4	20.9	21.0
mf							
M201	16.6	16.6	18.7	20.1	20.8	22.0	22.7
M208	18.2	19.5	21.2	22.5	23.0	23.6	25.0
M209	16.6	18.4	19.8	22.0	23.1	23.7	25.1
wm							
M203	20.8	21.1	24.6	25.7	26.3	26.6	27.2
M204	21.3	21.2	22.6	23.7	24.4	25.9	26.8
M212	19.3	20.6	22.5	23.5	24.1	24.3	25.5
mm							
M202	20.7	22.7	24.5	26.0	25.8	26.2	27.6
M205	20.9	23.5	25.5	26.7	27.4	28.5	29.5
M206	20.2	21.6	24.1	25.8	26.1	26.6	27.4
M211	20.7	22.4	24.7	27.2	27.4	28.4	30.3
M213	20.7	22.7	24.3	26.0	26.3	27.3	28.8
M214	21.0	24.1	26.6	27.6	27.8	28.8	29.3
M215	18.1	20.7	23.4	24.5	25.3	26.3	26.7

Average

	5	**6**	**8**	**9**	**10**	**11**	**12**
wf	17.5	18.5	19.5	20.1	20.6	21.0	21.4
mf	17.1	18.2	19.9	21.5	22.3	23.1	24.3
wm	20.5	21.0	23.2	24.3	24.9	25.6	26.5
mm	20.3	22.5	24.7	26.3	26.6	27.4	28.5

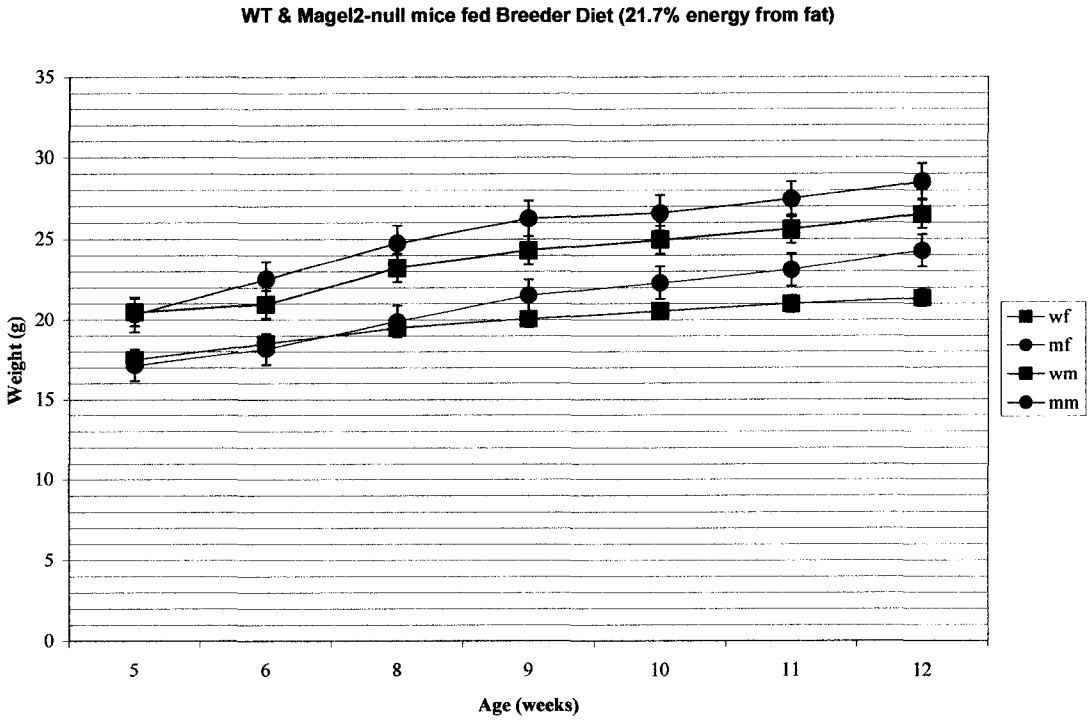


Figure 3.2 Wild type and *Magel2*-null mice fed a breeder diet. The data points are taken from the average weight for each group at each time point (see raw data at the bottom of Table 3.2). No data was recorded for week 7. wf = wild type female, mf = *Magel2*-null female, wm = wild type male, mm = *Magel2*-null male. Error bars are SEM (standard error of the mean).

Statistical analysis results for differences between wild type and *Magel2*-null at 6, 9, and 12 weeks are shown in Table 3.4. The results were statistically significant for the females at age 12 weeks and for the males at all ages. Full statistical analysis of each is detailed in Appendix A.

Table 3.4 T-test results for significant difference between wild type and *Magel2*-null mice fed a breeder diet at 6, 9, and 12 weeks. wf: wild type female, mf: *Magel2*-null female, wm: wild type male, mm: *Magel2*-null male. * = $p<0.05$.

	6	9	12
wf-mf	p=0.37	p=0.10	p=0.02*
wm-mm	p=0.01*	p=0.02*	p=0.02*

HIGH FAT DIET

Four litters were weaned and then fed a high fat diet (60.0% energy from fat). The breakdown of sex and genotype is as follows: wf = 9, mf = 5, wm = 10, and mm = 5. Table 3.3 shows the weight measurements over the weeks collected. Figure 3.3 shows the average of each group (e.g. wf) at each time point plotted against the other groups.

Table 3.5 Weight measurements of mice fed a high fat diet (60.0% energy from fat) from 4 weeks to 12 weeks of age. Blank boxes indicate no weight measurement taken. The average of each group at each time point is at the bottom. Some of this data was collected by J. Bischof.

	Age (weeks)								
wf	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
M264	18.2	19.9	22.7	23.4	24.2	27.4	27.0	28.5	29.5
M266	15.3	17.9	19.5	20.2	22.1	22.6	22.3	23.2	25.2
M279	14.9	17.6	21.1	21.5	23.0	24.4	25.5	26.7	28.0
M280	15.9	18.1	22.2	24.5	25.7	27.4	28.4	29.3	30.8
M288		17.4	18.2	19.5	21.2	21.6	23.8	24.4	27.5
M289		18.7	19.5	20.9	22.4	24.6	24.9	25.0	26.5
M291		16.8	17.7	18.4	19.9	20.8	21.7	23.0	24.1
M293		17.5	18.6	19.3	20.8	23.2	25.3	25.5	26.1
M297	16.0	17.9	19.5	22.0	22.6	23.1	24.7	25.8	27.5
mf									
M265	15.7	18.6	21.2	22.5	23.8	24.5	24.3	25.0	25.6
M281	12.1	15.1	17.7	19.2	20.3	22.5	23.2	25.1	27.4
M290		17.3	18.3	19.1	21.6	23.1	24.9	26.0	27.2
M292		15.8	16.8	18.0	19.7	21.2	22.3	22.8	23.6
M296	13.4	15.8	16.7	18.4	19.3	20.2	21.3	22.0	23.0
wm									
M267	21.0	23.5	25.8	27.2	29.9	30.7	33.5	34.5	35.9
M269	21.4	24.6	27.6	29.9	33.0	36.3	38.4	40.6	43.8
M283	18.6	21.8	24.8	26.8	28.3	31.1	33.0	34.9	37.0
M284	15.3	18.8	21.6	23.6	24.9	27.7	29.0	30.2	31.7
M285	17.9	20.6	22.9	25.2	27.6	29.5	30.5	31.4	32.3
M298	19.0	20.8	23.0	25.2	26.8	27.9	29.7	30.8	33.4
M299	16.0	17.5	19.1	21.7	23.5	24.6	25.2	25.8	27.9
M301	17.8	20.2	21.2	24.0	27.4	28.6	30.6	33.0	35.9
M302	17.6	19.4	20.9	23.7	26.0	27.1	28.5	30.8	32.4
M304	16.8	18.8	20.4	22.6	23.6	24.5	26.0	26.7	27.2
mm									
M268	17.2	19.0	21.0	21.6	24.1	26.0	26.6	27.6	29.0
M282	15.7	19.4	22.1	23.5	24.6	26.9	27.6	28.3	29.1
M286	15.2	18.5	20.6	22.5	24.9	27.2	28.2	29.2	30.5
M287	15.5	18.9	21.6	24.1	25.6	27.9	29.0	30.0	31.3
M303	13.5	17.2	19.1	20.7	22.4	23.6	25.1	26.0	27.1

Average

	4	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
wf	16.1	18.0	19.9	21.1	22.4	23.9	24.8	25.7	27.2
mf	13.7	16.5	18.1	19.4	20.9	22.3	23.2	24.2	25.4
wm	18.1	20.6	22.7	25.0	27.1	28.8	30.4	31.9	33.8
mm	15.4	18.6	20.9	22.5	24.3	26.3	27.3	28.2	29.4

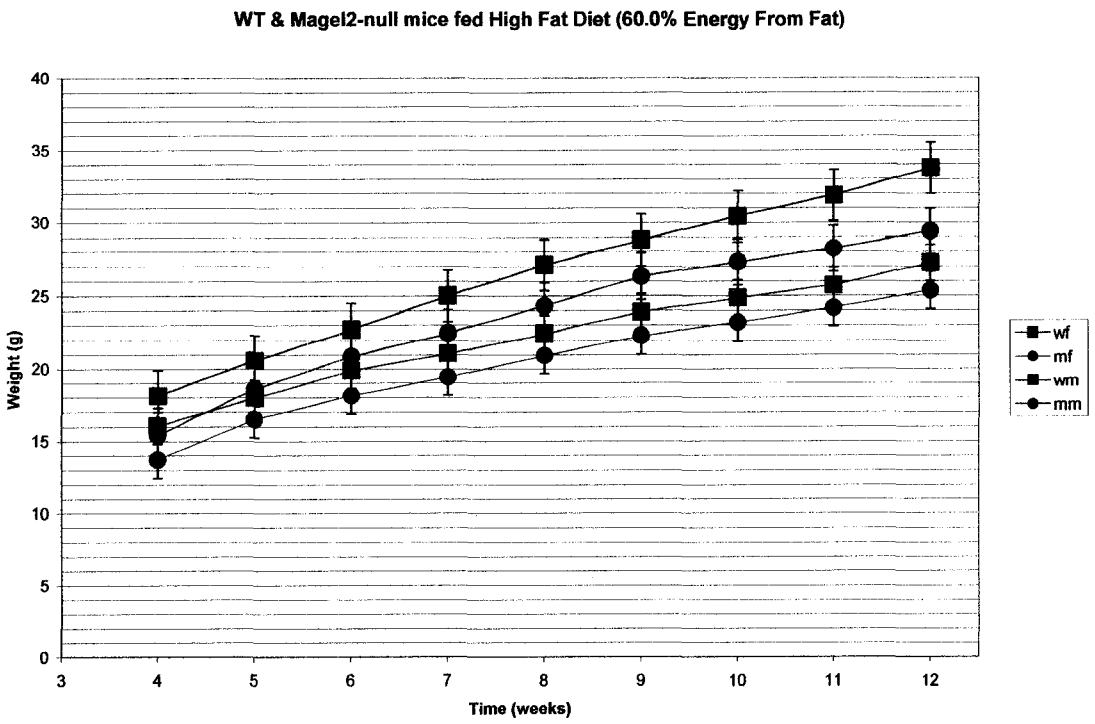


Figure 3.3 Wild type and *Magel2*-null mice fed a high fat diet. The data points are taken from the average weight for each group at each time point (see raw data at the bottom of Table 3.3). wf = wild type female, mf = *Magel2*-null female, wm = wild type male, mm = *Magel2*-null male. Error bars are SEM (standard error of the mean).

Statistical analysis results for differences between wild type and *Magel2*-null at 6, 9, and 12 weeks are shown in Table 3.6. The results were statistically significant for the females at age 6 weeks and for the males at age 12 weeks. Full statistical analysis of each is detailed in Appendix A.

Table 3.6 T-test results for significant difference between wild type and *Magel2*-null mice fed a high fat diet at 6, 9, and 12 weeks. wf: wild type female, mf: *Magel2*-null female, wm: wild type male, mm: *Magel2*-null male. * = $p<0.05$.

	6	9	12
wf-mf	p=0.05*	p=0.10	p=0.06
wm-mm	p=0.08	p=0.08	p=0.01*

BREEDING EXPERIMENTS

Two wild type mice and two *Magel2*-null mice, all approximately 3 ½ months old, were set up to breed with three females for one week. The males were left in a cage with three females for one week to ensure that all the females are receptive to breeding at least once during that week, as female mice are fertile every four days.

Breeding Experiment #1

The breeding set-up and resulting births (or lack of) are shown in Table 3.7. The wild type mice were able to impregnate four of the six females. The *Magel2*-null mice were not able to impregnate any of the females.

Table 3.7 Mating experiment #1. Two W.T. and two *Magel2*-null mice were independently set up to breed with three virgin females. The females were monitored for the birth of litters. Note that the *Magel2*-null mice did not produce any litters.

Mating Experiment 1

Cage1:	*Females:*	C420, C421, C422	Mating for 1 week; 08.03.06-15.03.06
	Male:	M203 (W.T.)	
Cage 2:	*Females:*	C423, C424, C425	Mating for 1 week; 08.03.06-15.03.06
	Male:	M204 (W.T.)	
Cage 3:	*Females:*	C449, C450, C451	Mating for 1 week; 08.03.06-15.03.06
	Male:	M205 (mt)	
Cage 4:	*Females:*	C452, C453, C454	Mating for 1 week; 08.03.06-15.03.06
	Male:	M214 (mt)	

Female	Birth Date - Pup #
C420	28.03.06 - 9
C421	30.03.06 - 9
C422	
C423	31.03.06 - 7
C424	
C425	28.03.06 - 6

Female	Birth Date - Pup #
C449	
C450	
C451	
C452	
C453	
C454	

Fisher Exact Probability Test (one-tailed)

	Not pregnant	Yes Pregnant
***Magel2*-null**	6	0
W.T.	2	4

$p = 0.03$

Therefore, the result of the *Magel2*-null males not being able to produce any offspring is significant.

Breeding Experiment #2

Because the *Magel2*-null mice failed to produce any offspring in the first experiment, the breeding experiment was repeated. The females that were used in the first experiment, and that did not produce any litters, were used again in second experiment. The females that were mated with either M205 or M214, were mated again, however, these mice were not paired up with the same male as they were in the first experiment. This was done to ensure that the lack of pregnancy/birth in the first experiment was not a problem with the female. The breeding set up and resulting births (or lack of) are shown in Table 3.8. The wild type mice were able to impregnate five of the six females and the Magel2-null mice were not able to impregnate any of the females.

Table 3.8 Mating experiment #2. The same two W.T. and two *Magel2*-null mice were independently set up to breed with three virgin females, some of which were used in the previous experiment. The females were monitored for the birth of litters. Note that again, the *Magel2*-null mice did not produce any litters.

Mating Experiment 2

Cage1:	*Females:*	C449, C452, C445	Mating for 1 week; 06.04.06-13.04.06
	Male:	M203 (W.T.)	
Cage 2:	*Females:*	C450, C453, C446	Mating for 1 week; 06.04.06-13.04.06
	Male:	M204 (W.T.)	
Cage 3:	*Females:*	C454, C422, C447	Mating for 1 week; 06.04.06-13.04.06
	Male:	M205 (mt)	
Cage 4:	*Females:*	C451, C424, C448	Mating for 1 week; 06.04.06-13.04.06
	Male:	M214 (mt)	

Female	Birth Date - Pup #
C449	30.04.06 - 9
C452	27.04.06 - 5
C445	29.04.06 - 8
C450	
C453	29.04.06 - 6
C446	29.04.06 - 9

Female	Birth Date - Pup #
C454	
C422	
C447	
C451	
C424	
C448	

Fisher Exact Probability Test (one-tailed)

	Not pregnant	Yes Pregnant
***Magel2*-null**	6	0
W.T.	1	5

$p = 0.0075$

Again, the result of the *Magel2*-null males not being able produce any offspring is significant. The second experiment gives a smaller p-value, indicating that the results of the second experiment show even more significance than the results of the first experiment.

Since breeding experiment #1 and breeding experiment #2 were done exactly the same way (i.e. #2 is a duplicate or repeat of #1, done four weeks later), the results of the two experiments can be combined. Using a Fisher exact probability test (one-tailed) to

analyze the combined results should strengthen the statistical output as the values have increased.

Fisher Exact Probability Test (one-tailed) – Combined results from Experiment #1 and Experiment #2.

	Not pregnant	**Yes Pregnant**
***Magel2*-null**	12	0
W.T.	3	9

$p = 0.00017$, or $p < 0.0002$

Combining the results of both experiments proves that the results of these experiments are highly significant, as evidenced by the extremely small p-value.

The results here prompted the next experiment: post mortem pathology of the testes and gonadal tissue, as well as measurements of serum testosterone levels in these mice. The results of this are outlined in the next section.

NECROPSY MEASUREMENTS

Post mortem pathology was performed on two wild type mice (M203 and M204) and on two *Magel2*-null mice (M205 and M214). The tissues weighed were brain, kidney, heart, liver, testicular fat pads, peri-renal fat, and subcutaneous fat (for M203 and M205 only). The weights of these tissues are shown in Table 3.9.

Table 3.9 Weight measurements for organs and tissues of two wild type mice and two *Magel2*-null mice. M203 and M204 are wild type. M205 and M214 are *Magel2*-null.

M203	Tissue	Weight
	Brain	0.434
	Kidney	0.332
	Heart	0.126
	Liver	1.217
	Testicular Fat Pads	1.034
	Perirenal Fat	0.283
	Subcutaneous fat/skin	5.176

M204	Tissue	Weight
	Brain	0.457
	Kidney	0.318
	Heart	0.121
	Liver	1.324
	Testicular Fat Pads	0.561
	Perirenal Fat	0.3

M205	Tissue	Weight
	Brain	0.438
	Kidney	0.324
	Heart	0.096
	Liver	1.463
	Testicular Fat Pads	2.08
	Perirenal Fat	0.818
	Subcutaneous fat/skin	7.785

M214	Tissue	Weight
	Brain	0.424
	Kidney	0.432
	Heart	0.176
	Liver	1.331
	Testicular Fat Pads	0.733
	Perirenal Fat	0.23

Because of the small sample size of necropsied mice, additional data was collected by a summer student, Joanna Cheung. Four additional *Magel2*-null mice and one additional wild type mouse were euthanized and the testicular fat and perirenal fat from each was weighed. The results and statistical analysis are shown in Tables 3.10 and 3.11.

Table 3.10 Testicular fat weight analysis between wild type and *Magel2*-null mice. wm: wild type males, mm: *Magel2*-null males.

wm	mm
1.03	2.08
0.56	0.73
1.60	2.33
	3.20
	3.24
	3.69

wm		*mm*	
Mean	1.06	Mean	2.55
Standard Error	0.30	Standard Error	0.44
Median	1.03	Median	2.77
Mode		Mode	
Standard Deviation	0.52	Standard Deviation	1.08
Sample Variance	0.27	Sample Variance	1.16
Kurtosis		Kurtosis	0.58
Skewness	0.29	Skewness	-0.96
Range	1.04	Range	2.96
Minimum	0.56	Minimum	0.73
Maximum	1.60	Maximum	3.69
Sum	3.19	Sum	15.27
Count	3.00	Count	6.00

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	1.06	2.55
Variance	0.27	1.16
Observations	3.00	6.00
Pooled Variance	0.90	
Hypothesized Mean Difference	0.00	
df	7.00	
t Stat	-2.20	
P(T<=t) one-tail	0.03	
t Critical one-tail	1.89	
P(T<=t) two-tail	0.06	
t Critical two-tail	2.36	

because the ratio between the SE's is less than two (1.46), need to use a t-test: two-sample assuming equal variance

Table 3.11 Perirenal fat weight analysis between wild type and *Magel2*-null mice. wm: wild type males, mm: *Magel2*-null males.

wm	mm
0.28	0.82
0.30	0.23
0.52	0.73
	0.81
	1.28
	1.40

wm		*mm*	
Mean	0.366667	Mean	0.878333
Standard Error	0.076884	Standard Error	0.171647
Median	0.3	Median	0.815
Mode		Mode	
Standard Deviation	0.133167	Standard Deviation	0.420448
Sample Variance	0.017733	Sample Variance	0.176777
Kurtosis		Kurtosis	-0.03876
Skewness	1.688202	Skewness	-0.28065
Range	0.24	Range	1.17
Minimum	0.28	Minimum	0.23
Maximum	0.52	Maximum	1.4
Sum	1.1	Sum	5.27
Count	3	Count	6

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	0.366667	0.878333
Variance	0.017733	0.176777
Observations	3	6
Hypothesized Mean Difference	0	
df	7	
t Stat	-2.720481	
P(T<=t) one-tail	0.014872	
t Critical one-tail	1.894579	
P(T<=t) two-tail	0.029745	
t Critical two-tail	2.364624	

because the ratio of the SE's is greater than two (2.23), then need to use t-test: two-sample assuming unequal variance

The difference between the wild type weights and the *Magel2*-null weights are significant for both the testicular and perirenal fat (p=0.03 and p=0.01, respectively). However, there was no significant differences in the weight of the organs (see Table 3.9).

The normal range of serum testosterone levels for an adult male mouse is 40 to 44,400 pg/mL (54). The wide variation in serum testosterone levels can be attributed to the fluctuation of testosterone release throughout the day and the antibody used for the test. The test will differ somewhat depending on the laboratory utilized for the testing. Serum from M203, M204, and M205 were tested for testosterone. M214 was not tested due to an insufficient amount of serum. The wild type mice, M203 and M204, had levels of 1070.0 pg/mL and 2885.0 pg/mL, respectively. The *Magel2*-null mouse, M205, had a level of 766.0 pg/mL. These levels are within the normal range.

The pathology done by Dr. Nick Nation on the testes of the wild type and *Magel2*-null mice failed to reveal any gross abnormalities. This result also fails to provide a reason for the inability of the *Magel2*-null males to impregnate any females.

DISCUSSION

For the mice that were fed a conventional diet, there is no significant difference between the weight of the wild type mice and the *Magel2*-null mice for both males and females. For mice that were fed the breeder's diet, the *Magel2*-null females are significantly heavier than the wild type females by the age of 12 weeks. For the male mice that were fed the breeder diet, the results indicate that there is a significant difference in the weight

of the *Magel2*-null mice versus the wild type mice at all the ages analyzed (6, 9 and 12 weeks). For the females being fed the high fat diet (60% energy from fat), there is a significant difference between the *Magel2*-null mice and the wild type mice at the age of 6 weeks. However, this result actually shows that the wild type mice are heavier than the *Magel2*-null mice. This significance disappears with time. For the male mice being fed the high fat diet, there is a significant difference between the wild type mice and the *Magel2*-null mice at 12 weeks. However, as with the females, this difference is actually that the wild type mice are heavier than the *Magel2*-null mice. For the experiments using all three diets, the number of mice in each sample is relatively small. Therefore, the results of these experiments need to be verified with an increased number of mice.

We would have predicted that the *Magel2*-null mice would weigh as much as the wild type mice when being fed the high fat diet. The reason for the reduced weight in the *Magel2*-null mice is not readily explained. A speculative reason behind this is that the diet is so rich in fat that the *Magel2*-null mice are unable to metabolize and utilize the food the same way as the wild type mice can.

Since there is a significant difference in the weight of the *Magel2*-null mice and the wild type mice that were fed the breeder diet, these mice were used for necropsy. The weight of the testicular fat and of the perirenal fat from each mouse was recorded and analyzed. The results show a significant difference in the weight of both the testicular and perirenal fat, with the weight being heavier in the *Magel2*-null mice. This result could suggest that in *Magel2*-null mice being fed a diet with a slightly higher fat content than normal, the

excess fat is retained and stored in the areas of the testes and around the kidneys. The significance of this is not well established, but it does rule out the difference in weight being due to a difference in the weight of the organs.

There was anecdotal evidence from our own experience as well as the experience of our collaborators that the *Magel2*-null mice were harder to breed. To test this anecdote, I devised a simple experiment to test the fertility of our *Magel2*-null males. The results of the experiment were very promising, in that none of the females mated with a *Magel2*-null male had offspring. This result prompted a repeat of this experiment. The repeat experiment resulted in identical data as the first experiment.

Testosterone levels and testes analysis on *Magel2*-null mice was conducted to see if either of these results could shed some light on to the apparent infertility of the *Magel2*-null male mice. The serum testosterone levels for the *Magel2*-null mouse fell into the normal range, albeit the lower end of the normal range. It remains to be determined if this is physiologically significant or not. The pathology of the testes failed to reveal any abnormalities that could explain the apparent infertility seen in the male *Magel2*-null mice. So far, it is known that there is a certain level of infertility in the *Magel2*-null male mice. It is also known at this point that there is no apparent abnormality in the testes and the serum testosterone level appears to be within normal range. An additional clue as to a possible cause of this infertility is the increase in testicular fat that is displayed by the *Magel2*-null mice. Therefore, there must be some other underlying cause of the apparent infertility in the *Magel2*-null males. There is also an experimental constraint that cannot

be ruled out as the cause of the apparent infertility. It is possible that the number of mice was too small, and a larger sample size would yield different results. Also, it is not know whether the mice actually mated, as it was not witnessed and there was no plug testing conducted. If the mice did mate, it is also possible that the female was actually pregnant, but had embryonic resorption. Another experimental constraint worth exploring is the age of the males, and if this affects their ability to impregnate the females in the given time period.

CHAPTER 4 ★ CONCLUSIONS AND FUTURE DIRECTIONS

MAGEL2 is a gene that is not actively expressed in PWS. Therefore, experiments were conducted to elucidate potential functions of *MAGEL2* in a normal situation. First, as *MAGEL2* is expressed in the hypothalamus, it seemed logical to conduct an experiment looking at the potential role of MAGEL2 in obesity and hyperphagia, since these are two symptoms that are characteristic of PWS and the hypothalamus is the predominant neurological center that controls these. Therefore, it seemed plausible that single gene mutations in *MAGEL2* could cause hyperphagia and obesity in non-PWS individuals. Secondly, mouse models are powerful tools in studying the effects of a lack of, or an over expression of a particular gene. A mouse lacking the *Magel2* gene was used in this research to analyze the effect on the mouse when *Magel2* is not expressed. Because of the specific and high expression of *Magel2* in the normal hypothalamus, it was expected that the *Magel2*-null mice would show abnormalities of the hypothalamus, such as hyperphagia/obesity, reproductive abnormalities, and dysfunction of the circadian rhythm.

OBESE AND HYPERPHAGIC DNA MUTATION ANALYSIS

There were no mutations identified in *MAGEL2* in the population of individuals studied. This population was based on two symptoms of PWS; childhood-onset obesity and hyperphagia. It is a distinct possibility that a mutation could be identified if the criteria for sample DNA were expanded to include individuals with childhood-onset obesity, hyperphagia, mental retardation and/or sleeping disorders. Despite there being no detrimental sequence alteration identified, there is still significance to the finding. The

result may eliminate *MAGEL2* as a candidate gene for obesity, or at least reduce its potency as a potential candidate gene for obesity.

A possible explanation for not finding a mutation in *MAGEL2* in this obese, hyperphagic population is that there could be a mutation outside of the ORF that affects the expression of *MAGEL2*. A mutation in the promoter region of the gene could affect expression through altered transcription factor binding. If this possibility does exist, it is important to note that this mutation would have to be in *cis* with the actively expressed allele; i.e. the paternal allele, since the maternal allele is silenced.

There are some known monogenic causes of obesity in humans (45). These include the MC4R (melanocortin-4 receptor) gene, as well as leptin and its receptor. Leptin is also known as *Ob*, from the obesity (*Ob/Ob*) mouse. However, these mutations only account for a minimal percentage of all obesity cases. Therefore, there must be other genes or combinations of genetic alleles that account for the remainder of obesity cases. The results of this research suggest that *MAGEL2* is *not* a gene responsible for monogenic, childhood-onset obesity with hyperphagia. However, since *MAGEL2* is not expressed in PWS, a quintessential obesity syndrome, it is likely that *MAGEL2* is involved in the dysfunction that leads to hyperphagia and obesity. As *NDN* is in the same family of proteins as *MAGEL2*, and it is also not expressed in PWS, and is also expressed in the hypothalamus, it is highly likely that the combined loss of both genes is responsible for the hyperphagia and obesity seen in PWS. Oeffner *et al* analyzed *NDN* for mutations in obese individuals, and did not find any conclusive results (51). Similarly, the results in

this research did not find any conclusive results indicating mutations in *MAGEL2* as being involved in obesity. Since both genes are not expressed in the obesity syndrome PWS, it certainly seems likely then that the combined loss of both genes is responsible for the hyperphagia and obesity seen in PWS. This also suggests that the loss of one gene is not enough to result in hyperphagia and obesity because the other gene is acting redundantly in the place of the inactive gene. Creation of a knockout mouse that is deficient for both *Ndn* and *Magel2* would likely provide the answer. However, there will be some technical difficulties in generating this knockout mice; it will have to be done in one of the mouse strains that has allowed *Ndn*-null mice to survive, and because *Ndn* is widely expressed in the nervous system, the knock-out of *Ndn* would have to conditional such that only hypothalamic expression of *Ndn* is terminated.

WEIGHT GAIN IN Magel2-null MICE VERSUS WILD TYPE MICE

The results of the weight gain analysis for the wild type and *Magel2*-null mice being fed the breeder diet indicate that *Magel2* is involved in obesity. However these results were not duplicated with either the conventional diet or the high fat diet. In fact, when the mice were fed the high fat diet, the wild type mice were significantly heavier than the *Magel2*-null mice. There is not a satisfactory explanation for this result, since the null hypothesis would be that there is no difference between the wild type mice and the *Magel2*-null mice. A reasonable explanation for the results of the experiment involving the high fat diet is that the *Magel2*-null mice have an inability to absorb the high fat content. If could be that the 60% energy from fat diet is simply too rich in fat and the gastrointestinal system in the *Magel2*-null mice is different enough from that in wild type

mice, such that there is a difference in energy uptake from the high fat diet. Exploration of the enteric nervous system of the *Magel2*-null mice will confirm or refute the notion that the null mice have an aberrant gastrointestinal system.

Based on the experiments done with the *Magel2*-null mice to assess their reproductive ability, it can be concluded that there is a serious deficit in reproductive ability. However, the experiments conducted and reported on in this thesis are unable to explain the infertility issues with the *Magel2*-null mice. The serum testosterone level for the *Magel2*-null mouse fell within the normal range. The histopathological analysis of the testes also failed to reveal any abnormalities that could explain the apparent infertility of the *Magel2*-null mice. The small sample size of the mice tested is a severe limitation of this experiment. More conclusive evidence would be obtained using a much larger sample size. Another limitation may be the age of the mice. The mice used in this experiment were approximately 15 weeks old, which under wild type circumstances is well within range for normal male reproductive ability; however, it could be that the *Magel2*-null mice have a much smaller window of fertility, ending before 15 weeks of age.

Further experiments were done in the Wevrick laboratory to assess *Magel2*-null male mice and their reproductive ability (J. Bischof). A breeding experiment was conducted; similar to the experiments I conducted, with the notable difference of a longer period of time where the male is in the cage with the females. In my experiments, I had left the male in the cage with the females for a period of one week, which is adequate time for

each female to go into estrous at least once. I was unable to get the *Magel2*-null males to produce any offspring. The newer experiments had the males paired with the females for a time period of two weeks. In this case the *Magel2*-null mice were able to produce offspring. However, the experiment also measured the time interval between pairing and mating. In the wild type mice, the average time period between pairing and mating was 3 days. The *Magel2*-null mice had an average time period between pairing and mating of 8.5 days, which is almost three times as long as the wild type time period. So in fact, the *Magel2*-null mice are capable of producing offspring, provided there is an extended period of mating. In light of these experiments, it is clear that the reproductive abnormality in *Magel2*-null male mice is not a drastic as the results of my experiments suggest. However, these experiments imply that there is still a reproductive abnormality in these mice; a timing dysfunction. The root cause of this timing defect is unknown, and is worth investigating further. Further testing of testosterone levels was also conducted (J. Bischof), which has given inconsistent results, even in the wild type samples. To get a more accurate measurement of hormone levels in the mice, the serum will be tested for luteinising hormone instead of testosterone, as testosterone levels are constantly cycling. Luteinising hormone is the hormone that stimulates testosterone production, and it is much more stable, and therefore an accurate assessment of testosterone level.

As outlined in Chapter 1, results from the Stewart laboratory indicate that *Magel2* is involved in circadian rhythm. This conclusion is based on their experiments with the *Magel2*-null mice in different light-dark situations. The *Magel2*-null mice exhibit a relatively normal pattern of activity in 12 hour light-12 hour dark cycles, and a

significantly non-consolidated pattern when released into complete darkness. This pattern of activity in light-dark, and in darkness, indicates a defect in the circadian rhythm output, rather than input, as the mice seem to have the ability to entrain to light.

Taking in to account all of the results discussed above, there is a strong indication to the involvement of the hypothalamus. This hypothalamic involvement becomes evident in the absence of *Magel2* expression. *Magel2* has significantly high expression in the hypothalamus. Therefore, a defect in the hypothalamus, due to the absence of *Magel2*, is responsible for being prone to obesity, reduced ability to breed, and a circadian rhythm defect.

Due to the small nature of the hypothalamus, with highly specific regions, it must be studied extremely closely. Cell numbers, cell size and cell types are all important avenues of research that need to be studied in the *Magel2*-null mice. Since there are regions in the hypothalamus with a finite area and a limited number of cells, it is quite conceivable that even a relatively small reduction in cell number could have a significant impact. Also, the distinct regions of the hypothalamus "talk" to each other through efferent projections. Therefore, it is possible that the cell types, numbers and size appear normal in the *Magel2*-null mice, but there is a lack of function due to absent or aberrant projections. The cells of the hypothalamus express specific neurotransmitters and their respective receptors. A reduction in the expression of either neurotransmitter or receptor would result in a dysfunctional hypothalamus. An experiment to look at the number of cells would involve the use of antibodies in combination with DAPI (or some other

nucleus stain), on cryosections of brain tissue. Antibodies to hypothalamic proteins such as NPY, POMC, and α-MSH would allow specified regions of the hypothalamus to be examined to count the number of cells. In order to look at the projections of the hypothalamus, immunohistochemistry or RNA *in situ* hybridization would work well on cryosections of the brain. For example, axons project from the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) and run through the infundibulum, terminating near the posterior lobe of the pituitary. These axons release vasopressin and oxytocin. Therefore, antibodies or RNA probes would label these axons. When the axons are labelled, one can follow the axonal projections on the cryosections, and compare the wild type to the *Magel2*-null. To analyze neurotransmitter and neurotransmitter receptor expression in the *Magel2*-null and compare it to wild type, RT-PCR (real time PCR) would be most effective. Possible RT-PCR targets would be NPY and the NPY-receptor, and α-MSH and MC4R (Melanocortin-4 receptor).

Another valuable avenue of research to pursue is the role of *Magel2* in the circadian rhythm cycle. The circadian rhythm is well researched, and therefore it offers a logical path to study. Finding the role of *Magel2* in the maintenance of the circadian rhythm could potentially shed light on the role of *Magel2* in other areas of the hypothalamus. These other areas include the role of *Magel2* in obesity, reproduction, and development of the early hypothalamus. There are other mouse models that represent defects in the circadian rhythm, such as the *Per* mouse, *Clock* mouse, and *Bmal* mouse (55). It would be interesting to mate these mice, such that the *Per/Clock/Bmal* mutant gene is recessive

and the mutant *Magel2* allele is inherited paternally, and analyze any differences from either parent strain.

There are numerous avenues of research that will shed light on the role of *MAGEL2*. This includes sequencing the promoter region of the *MAGEL2* gene, which would then need to be analyzed for mutations in the childhood-onset obesity with hyperphagia population. Other experimental paths include studying the subcellular localization of MAGEL2, and determining what other proteins interact with MAGEL2. An experiment of interest involving the *Magel2*-null mice would be to analyze the effect of the absence on *Magel2* expression on axonal extension in hypothalamic neurons.

The experiments and results in this thesis do not fully answer the question of MAGEL2's role in PWS, but it does shed some light as to the direction of future experiments. It is quite obvious that defects in the hypothalamus are a major component of the characteristics of PWS. The results in these experiments indicate that when there is a lack of *MAGEL2* expression, there is a negative consequence on the hypothalamus.

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APPENDIX A

t-Test and histograms for Conventional diet group.

Cumulative data 4-12 weeks.

M187	16.1	18.4		18.7	19.9	20.2	22.1	22.2	23.0
wm									
M121			23.9		25.8	25.2	26.0	25.4	26.1
M123			24.3		25.4	24.2	25.5	25.5	25.8
M128			21.9		24.9	24.9	25.9	25.6	26.6
M129			22.3		24.6	24.5	24.9	24.9	25.4
M158		20.3	21.2	22.6	24.0	24.5	24.9	25.5	25.1
M189	19.7	21.7		23.7	24.6	25.6	26.7	26.3	27.1
mm									
M122			24.7		27.7	27.5	27.3	27.3	27.8
M130			24.3		27.1	27.4	28.0	27.8	29.4
M159		19.1	21.1	23.0	24.3	25.1	25.3	24.8	24.9
M160		19.4	21.3	22.5	23.3	22.6	21.9	22.5	23.6
M161		19.5	21.5	22.7	24.5	24.9	24.9	24.4	25.2
M188	18.7	21.2		21.6	22.1	22.9	24.6	24.1	25.1
M190	20.9	23.6		25.8	27.3	28.4	29.6	29.4	30.2

Variance and t-test, 6 weeks.

wt	6 wks	6 wks	mt
M117	19.2	20.1	**M118**
M119	20.7	18.5	**M124**
M125	18.1	17.9	**M153**
M126	19.2	17.1	**M155**
M154	18.9	16.7	**M157**
M156	17.6		**M183**
M184			**M186**
M185			**M187**

Descriptive Stats

Column1		*Column2*	
Mean	19.0	Mean	18.1
Standard Error	0.4	Standard Error	0.6
Median	19.1	Median	17.9
Mode	19.2	Mode	
Standard Deviatior	1.1	Standard Deviatic	1.3
Sample Variance	1.1	Sample Variance	1.8
Kurtosis	0.8	Kurtosis	0.5
Skewness	0.6	Skewness	0.9
Range	3.1	Range	3.4
Minimum	17.6	Minimum	16.7
Maximum	20.7	Maximum	20.1
Sum	113.7	Sum	90.3
Count	6.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	19.0	18.1
Variance	1.1	1.8
Observations	6.0	5.0
Pooled Variance	1.4	
Hypothesized Mean D	0.0	
df	9.0	
t Stat	1.2	
P(T<=t) one-tail	0.1	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.3	
t Critical two-tail	2.3	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

wm	6 wks	6 wks	mm
M121	23.9	24.7	**M122**
M123	24.3	24.3	**M130**
M128	21.9	21.1	**M159**
M129	22.3	21.3	**M160**
M158	21.2	21.5	**M161**
M189			**M188**
			M190

Descriptive Stats

Column1		*Column2*	
Mean	22.7	Mean	22.6
Standard Error	0.6	Standard Error	0.8
Median	22.3	Median	21.5
Mode		Mode	
Standard Deviatior	1.3	Standard Deviatic	1.8
Sample Variance	1.8	Sample Variance	3.1
Kurtosis	-2.4	Kurtosis	-3.1
Skewness	0.3	Skewness	0.6
Range	3.1	Range	3.6
Minimum	21.2	Minimum	21.1
Maximum	24.3	Maximum	24.7
Sum	113.6	Sum	112.9
Count	5.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

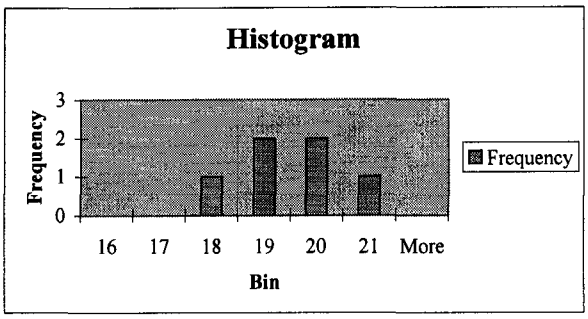
	Variable 1	*Variable 2*
Mean	22.7	22.6
Variance	1.8	3.1
Observations	5.0	5.0
Pooled Variance	2.4	
Hypothesized Mean D	0.0	
df	8.0	
t Stat	0.1	
P(T<=t) one-tail	0.4	
t Critical one-tail	1.9	
P(T<=t) two-tail	0.9	
t Critical two-tail	2.3	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

Histogram distribution, 6 weeks.

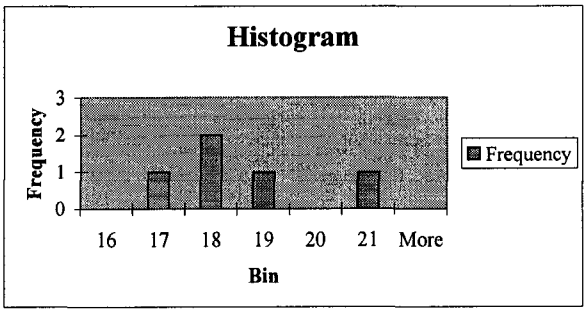
wt	6 wks	bin
M117	19.2	16
M119	20.7	17
M125	18.1	18
M126	19.2	19
M154	18.9	20
M156	17.6	21
M184		
M185		

Bin	*Frequency*
16	0
17	0
18	1
19	2
20	2
21	1
More	0



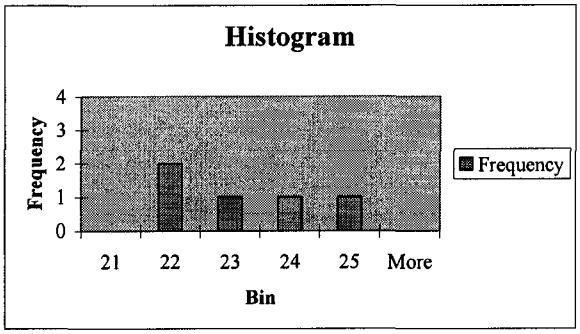
wt	6 wks	bin
M118	20.1	16
M124	18.5	17
M153	17.9	18
M155	17.1	19
M157	16.7	20
M183		21
M186		
M187		

Bin	*Frequency*
16	0
17	1
18	2
19	1
20	0
21	1
More	0



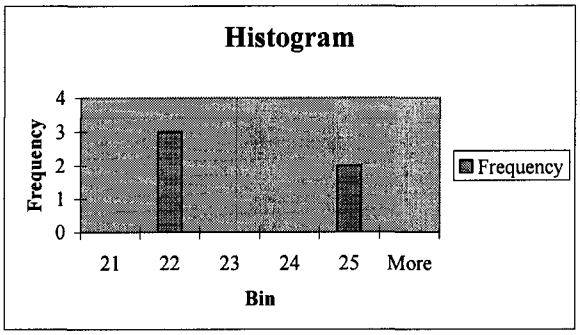
wt	6 wks	bin
M121	23.9	21
M123	24.3	22
M128	21.9	23
M129	22.3	24
M158	21.2	25
M189		

Bin	*Frequency*
21	0
22	2
23	1
24	1
25	1
More	0



wt	6 wks	bin
M122	24.7	21
M130	24.3	22
M159	21.1	23
M160	21.3	24
M161	21.5	25
M188		
M190		

Bin	*Frequency*
21	0
22	3
23	0
24	0
25	2
More	0



Variance and t-test, 9 weeks.

wf	9 wks	9 wks	mf
M117	20.4	23.3	**M118**
M119	22.2	21.1	**M124**
M125	20.0	22.4	**M153**
M126	20.3	17.2	**M155**
M154	21.0	17.2	**M157**
M156	18.9	18.9	**M183**
M184	20.3	18.0	**M186**
M185	21.7	20.2	**M187**

Descriptive Stats

Column1		*Column2*	
Mean	20.6	Mean	19.8
Standard Error	0.4	Standard Error	0.8
Median	20.4	Median	19.6
Mode	20.3	Mode	17.2
Standard Deviatic	1.0	Standard Deviatic	2.3
Sample Variance	1.1	Sample Variance	5.5
Kurtosis	0.2	Kurtosis	-1.5
Skewness	0.1	Skewness	0.3
Range	3.3	Range	6.1
Minimum	18.9	Minimum	17.2
Maximum	22.2	Maximum	23.3
Sum	164.8	Sum	158.3
Count	8.0	Count	8.0

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	20.6	19.8
Variance	1.1	5.5
Observations	8.0	8.0
Hypothesized Mean	0.0	
df	10.0	
t Stat	0.9	
P(T<=t) one-tail	0.2	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.4	
t Critical two-tail	2.2	

because the ratio of the SE's is greater than two (2.21), then need to use t-test: two-sample assuming unequal variance

wm	9 wks	9 wks	mm
M121	25.2	27.5	**M122**
M123	24.2	27.4	**M130**
M128	24.9	25.1	**M159**
M129	24.5	22.6	**M160**
M158	24.5	24.9	**M161**
M189	25.6	22.9	**M188**
		28.4	**M190**

Descriptive Stats

Column1		*Column2*	
Mean	24.8	Mean	25.5
Standard Error	0.2	Standard Error	0.9
Median	24.7	Median	25.1
Mode	24.5	Mode	
Standard Deviatic	0.5	Standard Deviatic	2.3
Sample Variance	0.3	Sample Variance	5.3
Kurtosis	-0.8	Kurtosis	-1.8
Skewness	0.5	Skewness	-0.1
Range	1.4	Range	5.8
Minimum	24.2	Minimum	22.6
Maximum	25.6	Maximum	28.4
Sum	148.9	Sum	178.8
Count	6.0	Count	7.0

t-Test: Two-Sample Assuming Unequal Variances

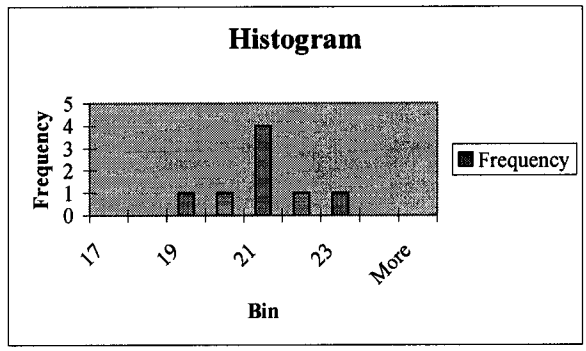
	Variable 1	*Variable 2*
Mean	24.8	25.5
Variance	0.3	5.3
Observations	6.0	7.0
Hypothesized Mean	0.0	
df	7.0	
t Stat	-0.8	
P(T<=t) one-tail	0.2	
t Critical one-tail	1.9	
P(T<=t) two-tail	0.4	
t Critical two-tail	2.4	

because the ratio of the SE's is greater than two (4.09), then need to use t-test: two-sample assuming unequal variance

Histogram distribution, 9 weeks.

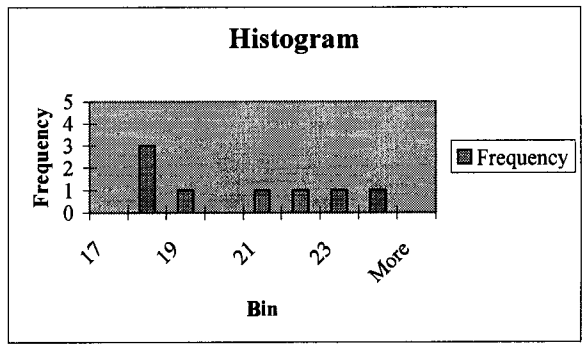
mf	9 wks	bin
M117	20.4	17
M119	22.2	18
M125	20.0	19
M126	20.3	20
M154	21.0	21
M156	18.9	22
M184	20.3	23
M185	21.7	24

Bin	*Frequency*
17	0
18	0
19	1
20	1
21	4
22	1
23	1
24	0
More	0



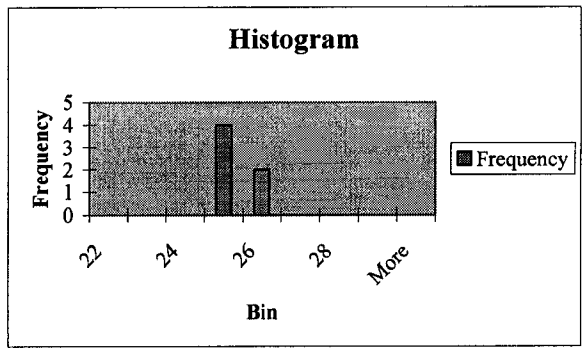
mf	9 wks	bin
M118	23.3	17
M124	21.1	18
M153	22.4	19
M155	17.2	20
M157	17.2	21
M183	18.9	22
M186	18.0	23
M187	20.2	24

Bin	*Frequency*
17	0
18	3
19	1
20	0
21	1
22	1
23	1
24	1
More	0



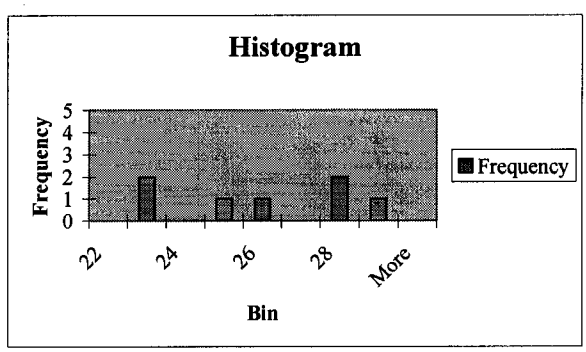
mf	9 wks	bin
M121	25.2	22
M123	24.2	23
M128	24.9	24
M129	24.5	25
M158	24.5	26
M189	25.6	27
		28
		29

Bin	*Frequency*
22	0
23	0
24	0
25	4
26	2
27	0
28	0
29	0
More	0



mf	9 wks	bin
M122	27.5	22
M130	27.4	23
M159	25.1	24
M160	22.6	25
M161	24.9	26
M188	22.9	27
M190	28.4	28
		29

Bin	*Frequency*
22	0
23	2
24	0
25	1
26	1
27	0
28	2
29	1
More	0



Variance and t-test, 12 weeks.

wf	12 wks	12 wks	mf
M117	21.3	24.4	**M118**
M119	24.9	21.1	**M124**
M125	20.7	21.1	**M153**
M126	21.4	18.2	**M155**
M154	22.0	18.4	**M157**
M156	19.7	21.9	**M183**
M184	21.1	20.4	**M186**
M185	24.1	23.0	**M187**

Descriptive Stats

Column1		*Column2*	
Mean	21.9	Mean	21.1
Standard Error	0.6	Standard Error	0.7
Median	21.4	Median	21.1
Mode		Mode	21.1
Standard Deviatio	1.7	Standard Deviatio	2.1
Sample Variance	3.1	Sample Variance	4.5
Kurtosis	-0.1	Kurtosis	-0.5
Skewness	0.9	Skewness	0.0
Range	5.2	Range	6.2
Minimum	19.7	Minimum	18.2
Maximum	24.9	Maximum	24.4
Sum	175.2	Sum	168.5
Count	8.0	Count	8.0

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	21.9	21.1
Variance	3.1	4.5
Observations	8.0	8.0
Pooled Variance	3.8	
Hypothesized Mea	0.0	
df	14.0	
t Stat	0.9	
P(T<=t) one-tail	0.2	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.4	
t Critical two-tail	2.1	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

wm	12 wks	12 wks	mm
M121	26.1	27.8	**M122**
M123	25.8	29.4	**M130**
M128	26.6	24.9	**M159**
M129	25.4	23.6	**M160**
M158	25.1	25.2	**M161**
M189	27.1	25.1	**M188**
		30.2	**M190**

Descriptive Stats

Column1		*Column2*	
Mean	26.0	Mean	26.6
Standard Error	0.3	Standard Error	1.0
Median	26.0	Median	25.2
Mode		Mode	
Standard Deviatio	0.7	Standard Deviatio	2.5
Sample Variance	0.6	Sample Variance	6.4
Kurtosis	-1.0	Kurtosis	-1.6
Skewness	0.3	Skewness	0.5
Range	2.0	Range	6.6
Minimum	25.1	Minimum	23.6
Maximum	27.1	Maximum	30.2
Sum	156.1	Sum	186.2
Count	6.0	Count	7.0

t-Test: Two-Sample Assuming Unequal Variances

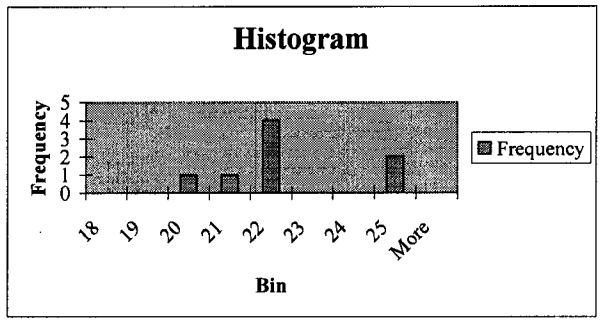
	Variable 1	*Variable 2*
Mean	26.0	26.6
Variance	0.6	6.4
Observations	6.0	7.0
Hypothesized Mea	0.0	
df	7.0	
t Stat	-0.6	
P(T<=t) one-tail	0.3	
t Critical one-tail	1.9	
P(T<=t) two-tail	0.6	
t Critical two-tail	2.4	

because the ratio of the SE's is greater than two (3.13), then need to use t-test: two-sample assuming unequal variance

Histogram distribution, 12 weeks.

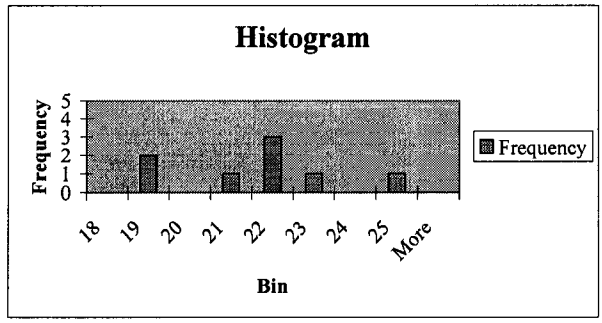
wf	12 wks	bin
M117	21.3	18
M119	24.9	19
M125	20.7	20
M126	21.4	21
M154	22.0	22
M156	19.7	23
M184	21.1	24
M185	24.1	25

Bin	*Frequency*
18	0
19	0
20	1
21	1
22	4
23	0
24	0
25	2
More	0



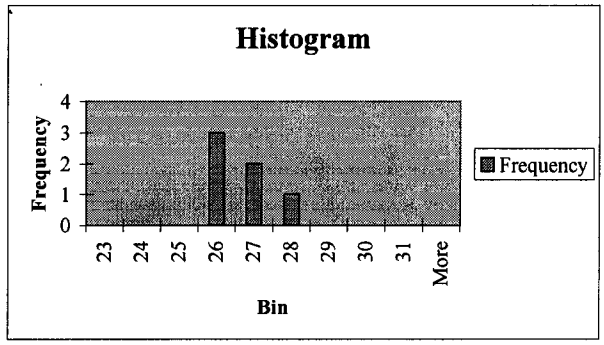
mf	12 wks	bin
M118	24.4	18
M124	21.1	19
M153	21.1	20
M155	18.2	21
M157	18.4	22
M183	21.9	23
M186	20.4	24
M187	23.0	25

Bin	*Frequency*
18	0
19	2
20	0
21	1
22	3
23	1
24	0
25	1
More	0



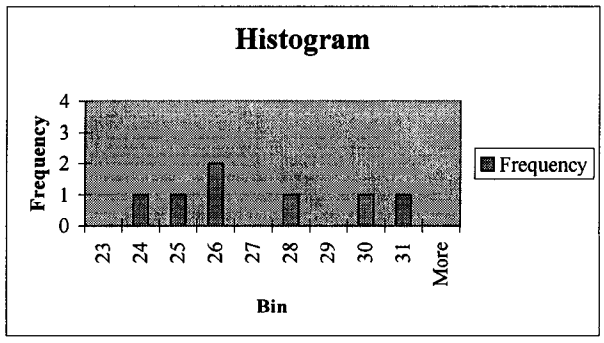
wm	12 wks	bin
M121	26.1	23
M123	25.8	24
M128	26.6	25
M129	25.4	26
M158	25.1	27
M189	27.1	28
		29
		30
		31

Bin	*Frequency*
23	0
24	0
25	0
26	3
27	2
28	1
29	0
30	0
31	0
More	0



mm	12 wks	bin
M122	27.8	23
M130	29.4	24
M159	24.9	25
M160	23.6	26
M161	25.2	27
M188	25.1	28
M190	30.2	29
		30
		31

Bin	*Frequency*
23	0
24	1
25	1
26	2
27	0
28	1
29	0
30	1
31	1
More	0



t-Test and histograms for Breeder diet group.

Cumulative data 5-12 weeks.

	Age (weeks)						
wf	5	6	8	9	10	11	12
M207	17.9	18.4	19.7	20.3	20.7	21.1	21.7
M210	17.1	18.6	19.3	19.8	20.4	20.9	21.0
mf							
M201	16.6	16.6	18.7	20.1	20.8	22.0	22.7
M208	18.2	19.5	21.2	22.5	23.0	23.6	25.0
M209	16.6	18.4	19.8	22.0	23.1	23.7	25.1
wm							
M203	20.8	21.1	24.6	25.7	26.3	26.6	27.2
M204	21.3	21.2	22.6	23.7	24.4	25.9	26.8
M212	19.3	20.6	22.5	23.5	24.1	24.3	25.5
mm							
M202	20.7	22.7	24.5	26.0	25.8	26.2	27.6
M205	20.9	23.5	25.5	26.7	27.4	28.5	29.5
M206	20.2	21.6	24.1	25.8	26.1	26.6	27.4
M211	20.7	22.4	24.7	27.2	27.4	28.4	30.3
M213	20.7	22.7	24.3	26.0	26.3	27.3	28.8
M214	21.0	24.1	26.6	27.6	27.8	28.8	29.3
M215	18.1	20.7	23.4	24.5	25.3	26.3	26.7

Average

	5	6	8	9	10	11	12
wf	17.50	18.50	19.50	20.05	20.55	21.00	21.35
mf	17.13	18.17	19.90	21.53	22.30	23.10	24.27
wm	20.47	20.97	23.23	24.30	24.93	25.60	26.50
mm	20.33	22.53	24.73	26.26	26.59	27.44	28.51

Variance and t-test, 6 weeks.

wt	6 wks	6 wks	trt
M207	18.4	16.6	**M201**
M210	18.6	19.5	**M208**
		18.4	**M209**

Descriptive stats

Column1		*Column2*	
Mean	18.5	Mean	18.2
Standard Error	0.1	Standard Error	0.8
Median	18.5	Median	18.4
Mode		Mode	
Standard Deviatio	0.1	Standard Deviatio	1.5
Sample Variance	0.0	Sample Variance	2.1
Kurtosis		Kurtosis	
Skewness		Skewness	-0.7
Range	0.2	Range	2.9
Minimum	18.4	Minimum	16.6
Maximum	18.6	Maximum	19.5
Sum	37.0	Sum	54.5
Count	2.0	Count	3.0

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	18.5	18.2
Variance	0.0	2.1
Observations	2.0	3.0
Hypothesized Mean	0.0	
df	2.0	
t Stat	0.4	
P(T<=t) one-tail	0.4	
t Critical one-tail	2.9	
P(T<=t) two-tail	0.7	
t Critical two-tail	4.3	

because the ratio of the SE's is greater than two (8.45), then need to use t-test: two-sample assuming unequal variance

wm	6 wks	6 wks	trt
M203	21.1	22.7	**M202**
M204	21.2	23.5	**M205**
M212	20.6	21.6	**M206**
		22.4	**M211**
		22.7	**M213**
		24.1	**M214**
		20.7	**M215**

Descriptive stats

Column1		*Column2*	
Mean	21.0	Mean	22.5
Standard Error	0.2	Standard Error	0.4
Median	21.1	Median	22.7
Mode		Mode	22.7
Standard Deviatio	0.3	Standard Deviatio	1.1
Sample Variance	0.1	Sample Variance	1.3
Kurtosis		Kurtosis	0.0
Skewness	-1.5	Skewness	-0.3
Range	0.6	Range	3.4
Minimum	20.6	Minimum	20.7
Maximum	21.2	Maximum	24.1
Sum	62.9	Sum	157.7
Count	3.0	Count	7.0

t-Test: Two-Sample Assuming Unequal Variances

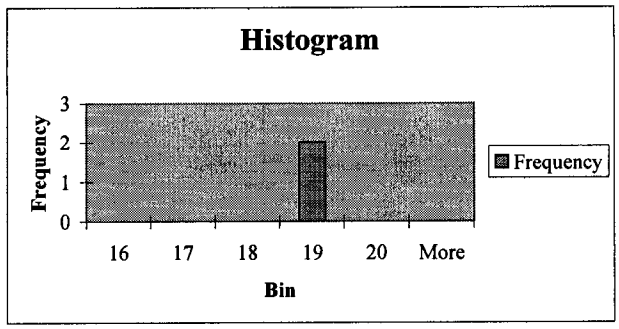
	Variable 1	*Variable 2*
Mean	21.0	22.5
Variance	0.1	1.3
Observations	3.0	7.0
Hypothesized Mean	0.0	
df	8.0	
t Stat	-3.3	
P(T<=t) one-tail	0.0	
t Critical one-tail	1.9	
P(T<=t) two-tail	0.0	
t Critical two-tail	2.3	

because the ratio of the SE's is greater than two (2.31), then need to use t-test: two-sample assuming unequal variance

Histogram distribution, 6 weeks.

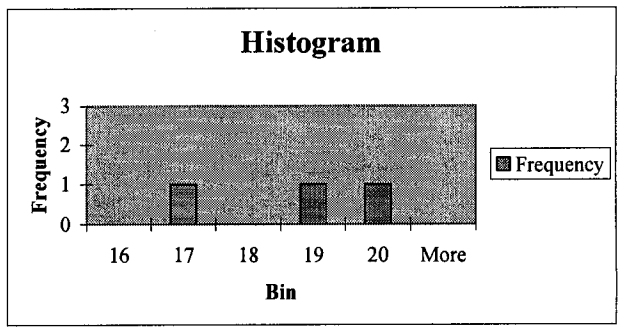
Rat	6 wks	bin
M207	18.4	16
M210	18.6	17
		18
		19
		20

Bin	*Frequency*
16	0
17	0
18	0
19	2
20	0
More	0



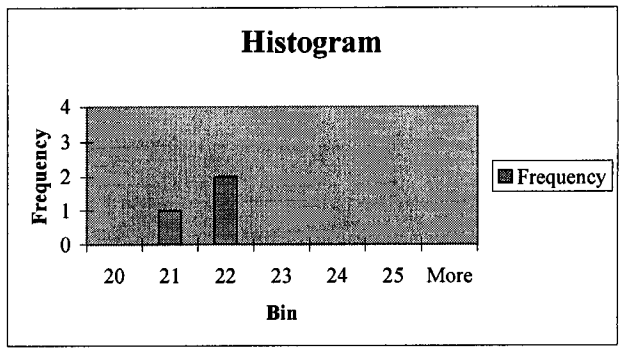
Rat	6 wks	bin
M201	16.6	16
M208	19.5	17
M209	18.4	18
		19
		20

Bin	*Frequency*
16	0
17	1
18	0
19	1
20	1
More	0



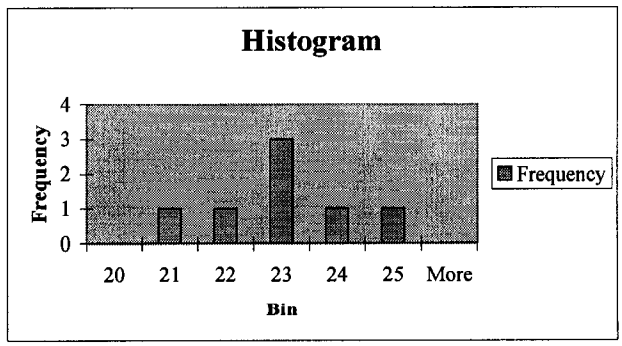
Rat	6 wks	bin
M203	21.1	20
M204	21.2	21
M212	20.6	22
		23
		24
		25

Bin	*Frequency*
20	0
21	1
22	2
23	0
24	0
25	0
More	0



Rat	6 wks	bin
M202	22.7	20
M205	23.5	21
M206	21.6	22
M211	22.4	23
M213	22.7	24
M214	24.1	25
M215	20.7	

Bin	*Frequency*
20	0
21	1
22	1
23	3
24	1
25	1
More	0



Variance and t-test, 9 weeks.

wks	9 wks	9 wks	mf
M207	20.3	20.1	**M201**
M210	19.8	22.5	**M208**
		22.0	**M209**

Descriptive stats

Column1		*Column2*	
Mean	20.1	Mean	21.5
Standard Error	0.3	Standard Error	0.7
Median	20.1	Median	22.0
Mode		Mode	
Standard Deviatio	0.4	Standard Deviatio	1.3
Sample Variance	0.1	Sample Variance	1.6
Kurtosis		Kurtosis	
Skewness		Skewness	-1.4
Range	0.5	Range	2.4
Minimum	19.8	Minimum	20.1
Maximum	20.3	Maximum	22.5
Sum	40.1	Sum	64.6
Count	2.0	Count	3.0

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	20.1	21.5
Variance	0.1	1.6
Observations	2.0	3.0
Hypothesized Mea	0.0	
df	2.0	
t Stat	-1.9	
P(T<=t) one-tail	0.1	
t Critical one-tail	2.9	
P(T<=t) two-tail	0.2	
t Critical two-tail	4.3	

because the ratio of the SE's is greater than two (2.92), then need to use t-test: two-sample assuming unequal variance

wm	9 wks	9 wks	mm
M203	25.7	26.0	**M202**
M204	23.7	26.7	**M205**
M212	23.5	25.8	**M206**
		27.2	**M211**
		26.0	**M213**
		27.6	**M214**
		24.5	**M215**

Descriptive stats

Column1		*Column2*	
Mean	24.3	Mean	26.3
Standard Error	0.7	Standard Error	0.4
Median	23.7	Median	26.0
Mode		Mode	26.0
Standard Deviatio	1.2	Standard Deviatio	1.0
Sample Variance	1.5	Sample Variance	1.1
Kurtosis		Kurtosis	0.4
Skewness	1.7	Skewness	-0.5
Range	2.2	Range	3.1
Minimum	23.5	Minimum	24.5
Maximum	25.7	Maximum	27.6
Sum	72.9	Sum	183.8
Count	3.0	Count	7.0

t-Test: Two-Sample Assuming Equal Variances

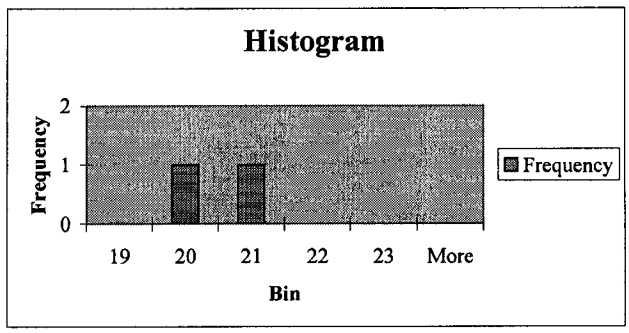
	Variable 1	*Variable 2*
Mean	24.3	26.3
Variance	1.5	1.1
Observations	3.0	7.0
Pooled Variance	1.2	
Hypothesized Mea	0.0	
df	8.0	
t Stat	-2.6	
P(T<=t) one-tail	0.0	
t Critical one-tail	1.9	
P(T<=t) two-tail	0.0	
t Critical two-tail	2.3	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

Histogram distribution, 9 weeks.

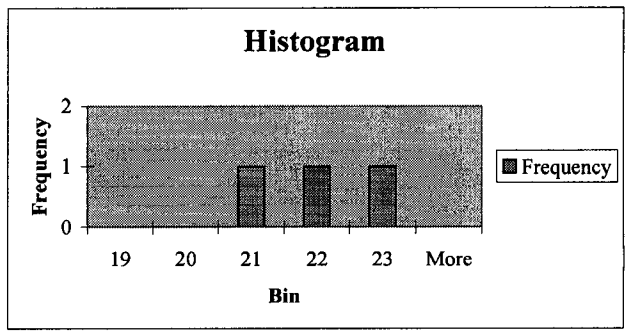
	9 wks	bin
M207	20.3	19
M210	19.8	20
		21
		22
		23

Bin	*Frequency*
19	0
20	1
21	1
22	0
23	0
More	0



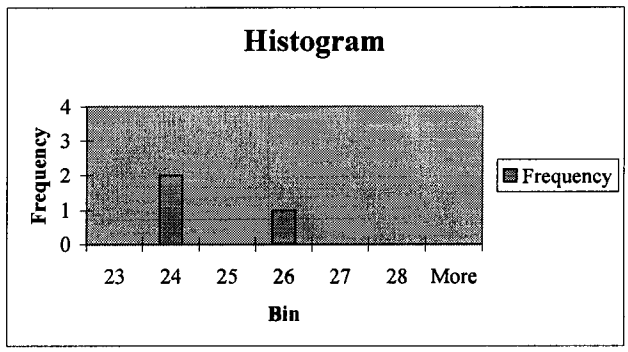
	9 wks	bin
M201	20.1	19
M208	22.5	20
M209	22.0	21
		22
		23

Bin	*Frequency*
19	0
20	0
21	1
22	1
23	1
More	0



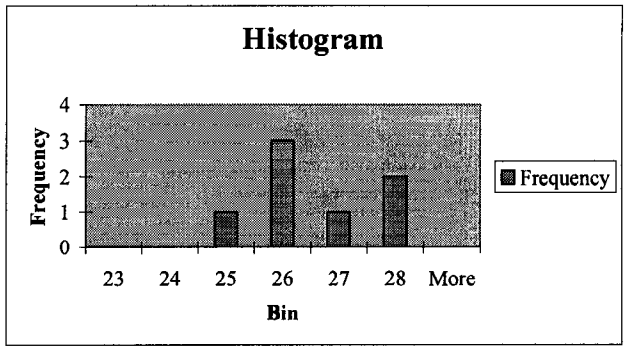
	9 wks	bin
M203	25.7	23
M204	23.7	24
M212	23.5	25
		26
		27
		28

Bin	*Frequency*
23	0
24	2
25	0
26	1
27	0
28	0
More	0



	9 wks	bin
M202	26.0	23
M205	26.7	24
M206	25.8	25
M211	27.2	26
M213	26.0	27
M214	27.6	28
M215	24.5	

Bin	*Frequency*
23	0
24	0
25	1
26	3
27	1
28	2
More	0



Variance and t-test, 12 weeks.

wt	12 wks	12 wks	mt
M207	21.7	22.7	**M201**
M210	21.0	25.0	**M208**
		25.1	**M209**

Descriptive stats

Column1		*Column2*	
Mean	21.4	Mean	24.3
Standard Error	0.3	Standard Error	0.8
Median	21.4	Median	25.0
Mode		Mode	
Standard Deviatio	0.5	Standard Deviatio	1.4
Sample Variance	0.2	Sample Variance	1.8
Kurtosis		Kurtosis	
Skewness		Skewness	-1.7
Range	0.7	Range	2.4
Minimum	21.0	Minimum	22.7
Maximum	21.7	Maximum	25.1
Sum	42.7	Sum	72.8
Count	2.0	Count	3.0

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	21.4	24.3
Variance	0.2	1.8
Observations	2.0	3.0
Hypothesized Mean	0.0	
df	3.0	
t Stat	-3.4	
P(T<=t) one-tail	0.0	
t Critical one-tail	2.4	
P(T<=t) two-tail	0.0	
t Critical two-tail	3.2	

because the ratio of the SE's is greater than two (2.24), then need to use t-test: two-sample assuming unequal variance

wm	12 wks	12 wks	mm
M203	27.2	27.6	**M202**
M204	26.8	29.5	**M205**
M212	25.5	27.4	**M206**
		30.3	**M211**
		28.8	**M213**
		29.3	**M214**
		26.7	**M215**

Descriptive stats

Column1		*Column2*	
Mean	26.5	Mean	28.5
Standard Error	0.5	Standard Error	0.5
Median	26.8	Median	28.8
Mode		Mode	
Standard Deviatio	0.9	Standard Deviatio	1.3
Sample Variance	0.8	Sample Variance	1.7
Kurtosis		Kurtosis	-1.5
Skewness	-1.3	Skewness	-0.1
Range	1.7	Range	3.6
Minimum	25.5	Minimum	26.7
Maximum	27.2	Maximum	30.3
Sum	79.5	Sum	199.6
Count	3.0	Count	7.0

t-Test: Two-Sample Assuming Equal Variances

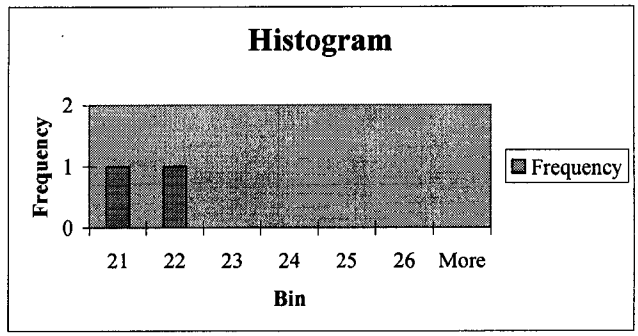
	Variable 1	*Variable 2*
Mean	26.5	28.5
Variance	0.8	1.7
Observations	3.0	7.0
Pooled Variance	1.5	
Hypothesized Mean	0.0	
df	8.0	
t Stat	-2.4	
P(T<=t) one-tail	0.0	
t Critical one-tail	1.9	
P(T<=t) two-tail	0.0	
t Critical two-tail	2.3	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

Histogram distribution, 12 weeks.

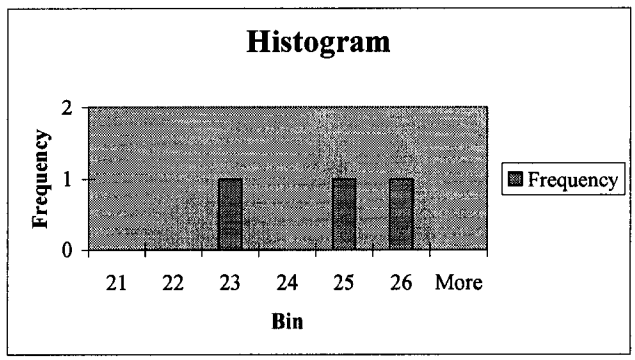
wt	12 wks	bin
M207	21.7	21
M210	21.0	22
		23
		24
		25
		26

Bin	*Frequency*
21	1
22	1
23	0
24	0
25	0
26	0
More	0



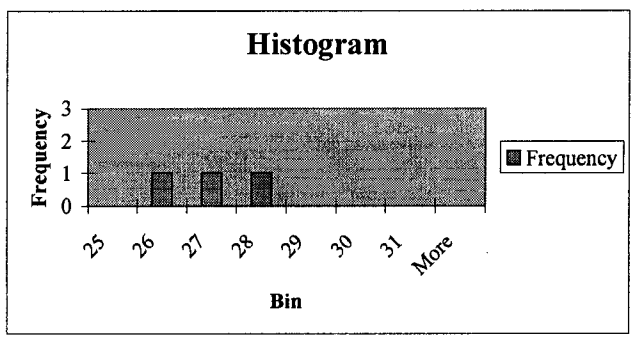
wt	12 wks	bin
M201	22.7	21
M208	25.0	22
M209	25.1	23
		24
		25
		26

Bin	*Frequency*
21	0
22	0
23	1
24	0
25	1
26	1
More	0



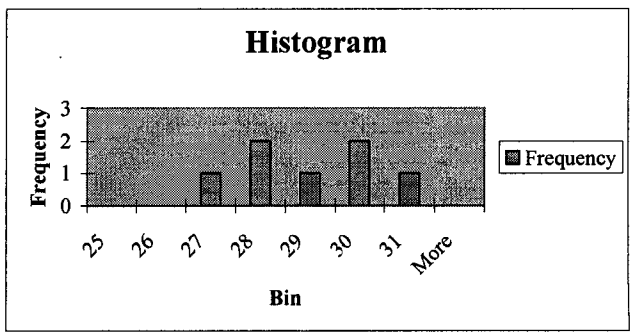
wt	12 wks	bin
M203	27.2	25
M204	26.8	26
M212	25.5	27
		28
		29
		30
		31

Bin	*Frequency*
25	0
26	1
27	1
28	1
29	0
30	0
31	0
More	0



wt	12 wks	bin
M202	27.6	25
M205	29.5	26
M206	27.4	27
M211	30.3	28
M213	28.8	29
M214	29.3	30
M215	26.7	31

Bin	*Frequency*
25	0
26	0
27	1
28	2
29	1
30	2
31	1
More	0



t-Test and histograms for 60% energy from fat diet group.

Cumulative data 4-12 weeks.

Age (weeks)

wf	4	5	6	7	8	9	10	11	12
M264	18.2	19.9	22.7	23.4	24.2	27.4	27.0	28.5	29.5
M266	15.3	17.9	19.5	20.2	22.1	22.6	22.3	23.2	25.2
M279	14.9	17.6	21.1	21.5	23.0	24.4	25.5	26.7	28.0
M280	15.9	18.1	22.2	24.5	25.7	27.4	28.4	29.3	30.8
M288		17.4	18.2	19.5	21.2	21.6	23.8	24.4	27.5
M289		18.7	19.5	20.9	22.4	24.6	24.9	25.0	26.5
M291		16.8	17.7	18.4	19.9	20.8	21.7	23.0	24.1
M293		17.5	18.6	19.3	20.8	23.2	25.3	25.5	26.1
M297	16.0	17.9	19.5	22.0	22.6	23.1	24.7	25.8	27.5
mf									
M265	15.7	18.6	21.2	22.5	23.8	24.5	24.3	25.0	25.6
M281	12.1	15.1	17.7	19.2	20.3	22.5	23.2	25.1	27.4
M290		17.3	18.3	19.1	21.6	23.1	24.9	26.0	27.2
M292		15.8	16.8	18.0	19.7	21.2	22.3	22.8	23.6
M296	13.4	15.8	16.7	18.4	19.3	20.2	21.3	22.0	23.0
wm									
M267	21.0	23.5	25.8	27.2	29.9	30.7	33.5	34.5	35.9
M269	21.4	24.6	27.6	29.9	33.0	36.3	38.4	40.6	43.8
M283	18.6	21.8	24.8	26.8	28.3	31.1	33.0	34.9	37.0
M284	15.3	18.8	21.6	23.6	24.9	27.7	29.0	30.2	31.7
M285	17.9	20.6	22.9	25.2	27.6	29.5	30.5	31.4	32.3
M298	19.0	20.8	23.0	25.2	26.8	27.9	29.7	30.8	33.4
M299	16.0	17.5	19.1	21.7	23.5	24.6	25.2	25.8	27.9
M301	17.8	20.2	21.2	24.0	27.4	28.6	30.6	33.0	35.9
M302	17.6	19.4	20.9	23.7	26.0	27.1	28.5	30.8	32.4
M304	16.8	18.8	20.4	22.6	23.6	24.5	26.0	26.7	27.2
mm									
M268	17.2	19.0	21.0	21.6	24.1	26.0	26.6	27.6	29.0
M282	15.7	19.4	22.1	23.5	24.6	26.9	27.6	28.3	29.1
M286	15.2	18.5	20.6	22.5	24.9	27.2	28.2	29.2	30.5
M287	15.5	18.9	21.6	24.1	25.6	27.9	29.0	30.0	31.3
M303	13.5	17.2	19.1	20.7	22.4	23.6	25.1	26.0	27.1

Average

	4	5	6	7	8	9	10	11	12
wf	16.06	17.98	19.89	21.08	22.43	23.90	24.84	25.71	27.24
mf	13.73	16.52	18.14	19.44	20.94	22.30	23.20	24.18	25.36
wm	18.14	20.60	22.73	24.99	27.10	28.80	30.44	31.87	33.75
mm	15.42	18.60	20.88	22.48	24.32	26.32	27.30	28.22	29.40

Variance and t-test, 6 weeks.

wf	6 wks	6 wks	mf
M264	22.7	21.2	**M265**
M266	19.5	17.7	**M281**
M279	21.1	18.3	**M290**
M280	22.2	16.8	**M292**
M288	18.2	16.7	**M296**
M289	19.5		
M291	17.7		
M293	18.6		
M297	19.5		

Descriptive stats

Column1		*Column2*	
Mean	19.9	Mean	18.1
Standard Error	0.6	Standard Error	0.8
Median	19.5	Median	17.7
Mode	19.5	Mode	
Standard Deviati	1.7	Standard Deviati	1.8
Sample Variance	3.1	Sample Variance	3.4
Kurtosis	-0.9	Kurtosis	2.5
Skewness	0.6	Skewness	1.6
Range	5.0	Range	4.5
Minimum	17.7	Minimum	16.7
Maximum	22.7	Maximum	21.2
Sum	179.0	Sum	90.7
Count	9.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	19.9	18.1
Variance	3.1	3.4
Observations	9.0	5.0
Pooled Variance	3.2	
Hypothesized Mea	0.0	
df	12.0	
t Stat	1.8	
P(T<=t) one-tail	0.1	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.1	
t Critical two-tail	2.2	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

wm	6 wks	6 wks	mm
M267	25.8	21.0	**M268**
M269	27.6	22.1	**M282**
M283	24.8	20.6	**M286**
M284	21.6	21.6	**M287**
M285	22.9	19.1	**M303**
M298	23.0		
M299	19.1		
M301	21.2		
M302	20.9		
M304	20.4		

Descriptive stats

Column1		*Column2*	
Mean	22.7	Mean	20.9
Standard Error	0.8	Standard Error	0.5
Median	22.3	Median	21.0
Mode		Mode	
Standard Deviati	2.6	Standard Deviati	1.1
Sample Variance	7.0	Sample Variance	1.3
Kurtosis	-0.4	Kurtosis	1.0
Skewness	0.6	Skewness	-1.0
Range	8.5	Range	3.0
Minimum	19.1	Minimum	19.1
Maximum	27.6	Maximum	22.1
Sum	227.3	Sum	104.4
Count	10.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

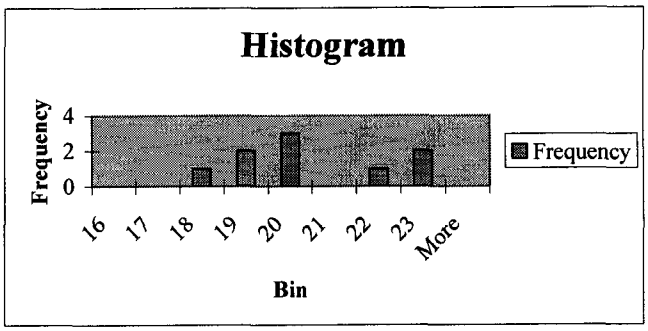
	Variable 1	*Variable 2*
Mean	22.7	20.9
Variance	7.0	1.3
Observations	10.0	5.0
Pooled Variance	5.3	
Hypothesized Mea	0.0	
df	13.0	
t Stat	1.5	
P(T<=t) one-tail	0.1	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.2	
t Critical two-tail	2.2	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

Histogram distribution, 6 weeks.

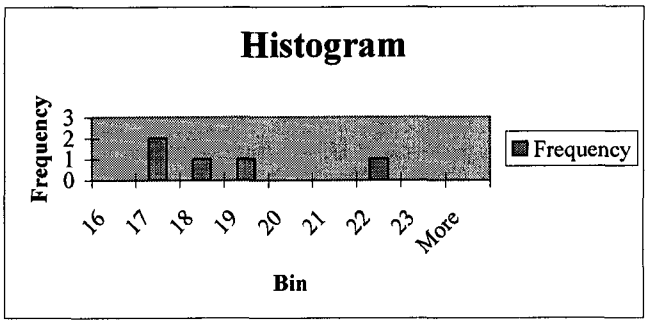
wf	6 wks	bin
M264	22.7	16
M266	19.5	17
M279	21.1	18
M280	22.2	19
M288	18.2	20
M289	19.5	21
M291	17.7	22
M293	18.6	23
M297	19.5	

Bin	Frequency
16	0
17	0
18	1
19	2
20	3
21	0
22	1
23	2
More	0



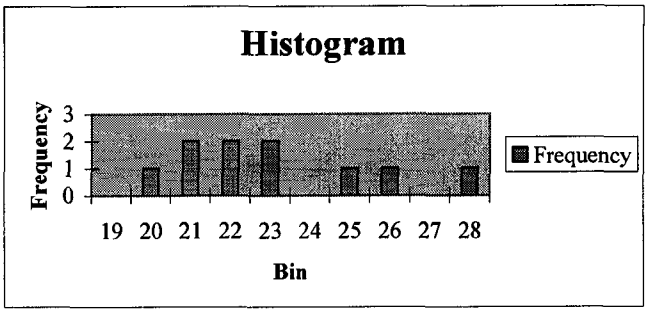
mf	6 wks	bin
M265	21.2	16
M281	17.7	17
M290	18.3	18
M292	16.8	19
M296	16.7	20
		21
		22
		23

Bin	Frequency
16	0
17	2
18	1
19	1
20	0
21	0
22	1
23	0
More	0



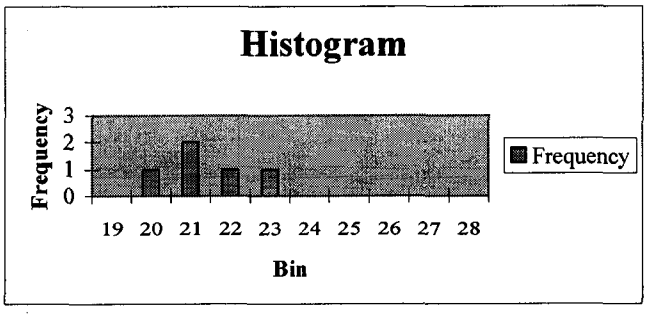
wm	6 wks	bin
M267	25.8	19
M269	27.6	20
M283	24.8	21
M284	21.6	22
M285	22.9	23
M298	23.0	24
M299	19.1	25
M301	21.2	26
M302	20.9	27
M304	20.4	28

Bin	Frequency
19	0
20	1
21	2
22	2
23	2
24	0
25	1
26	1
27	0
28	1



mm	6 wks	bin
M268	21.0	19
M282	22.1	20
M286	20.6	21
M287	21.6	22
M303	19.1	23
		24
		25
		26
		27
		28

Bin	Frequency
19	0
20	1
21	2
22	1
23	1
24	0
25	0
26	0
27	0
28	0



Variance and t-test, 9 weeks.

wf	9 wks	9 wks	mf
M264	27.4	24.5	**M265**
M266	22.6	22.5	**M281**
M279	24.4	23.1	**M290**
M280	27.4	21.2	**M292**
M288	21.6	20.2	**M296**
M289	24.6		
M291	20.8		
M293	23.2		
M297	23.1		

Descriptive stats

Column1		*Column2*	
Mean	23.9	Mean	22.3
Standard Error	0.8	Standard Error	0.7
Median	23.2	Median	22.5
Mode	27.4	Mode	
Standard Deviati	2.3	Standard Deviati	1.7
Sample Variance	5.4	Sample Variance	2.8
Kurtosis	-0.6	Kurtosis	-0.8
Skewness	0.5	Skewness	0.1
Range	6.6	Range	4.3
Minimum	20.8	Minimum	20.2
Maximum	27.4	Maximum	24.5
Sum	215.1	Sum	111.5
Count	9.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	23.9	22.3
Variance	5.4	2.8
Observations	9.0	5.0
Pooled Variance	4.5	
Hypothesized Mean	0.0	
df	12.0	
t Stat	1.4	
P(T<=t) one-tail	0.1	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.2	
t Critical two-tail	2.2	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

wm	9 wks	9 wks	mm
M267	30.7	26.0	**M268**
M269	36.3	26.9	**M282**
M283	31.1	27.2	**M286**
M284	27.7	27.9	**M287**
M285	29.5	23.6	**M303**
M298	27.9		
M299	24.6		
M301	28.6		
M302	27.1		
M304	24.5		

Descriptive stats

Column1		*Column2*	
Mean	28.8	Mean	26.3
Standard Error	1.1	Standard Error	0.7
Median	28.3	Median	26.9
Mode		Mode	
Standard Deviati	3.4	Standard Deviati	1.7
Sample Variance	11.9	Sample Variance	2.8
Kurtosis	1.7	Kurtosis	2.0
Skewness	1.0	Skewness	-1.4
Range	11.8	Range	4.3
Minimum	24.5	Minimum	23.6
Maximum	36.3	Maximum	27.9
Sum	288.0	Sum	131.6
Count	10.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

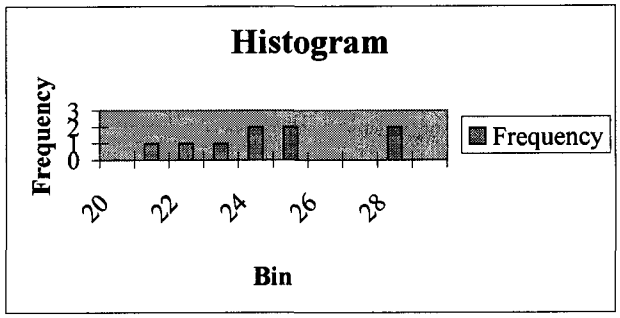
	Variable 1	*Variable 2*
Mean	28.8	26.3
Variance	11.9	2.8
Observations	10.0	5.0
Pooled Variance	9.1	
Hypothesized Mean	0.0	
df	13.0	
t Stat	1.5	
P(T<=t) one-tail	0.1	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.2	
t Critical two-tail	2.2	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

Histogram distribution, 9 weeks.

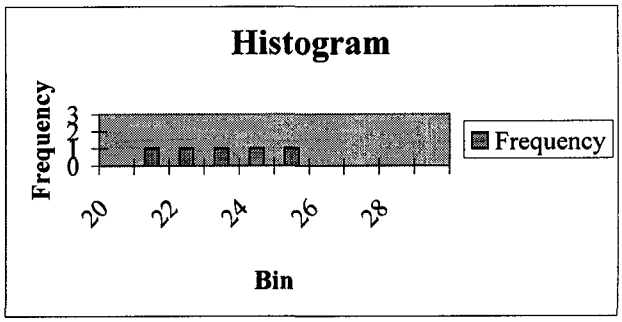
wt	9 wks	bin
M264	27.4	20
M266	22.6	21
M279	24.4	22
M280	27.4	23
M288	21.6	24
M289	24.6	25
M291	20.8	26
M293	23.2	27
M297	23.1	28

Bin	Frequency
20	0
21	1
22	1
23	1
24	2
25	2
26	0
27	0
28	2
More	0



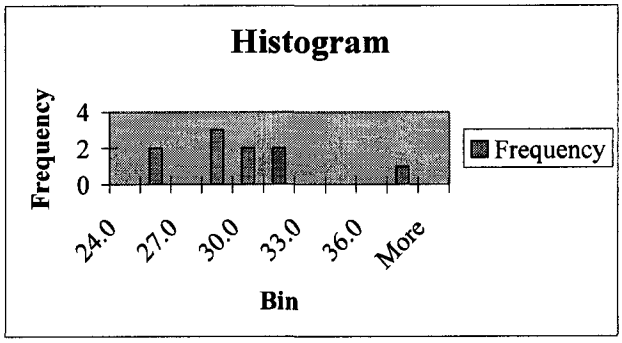
mt	9 wks	bin
M265	24.5	20
M281	22.5	21
M290	23.1	22
M292	21.2	23
M296	20.2	24
		25
		26
		27
		28

Bin	Frequency
20	0
21	1
22	1
23	1
24	1
25	1
26	0
27	0
28	0
More	0



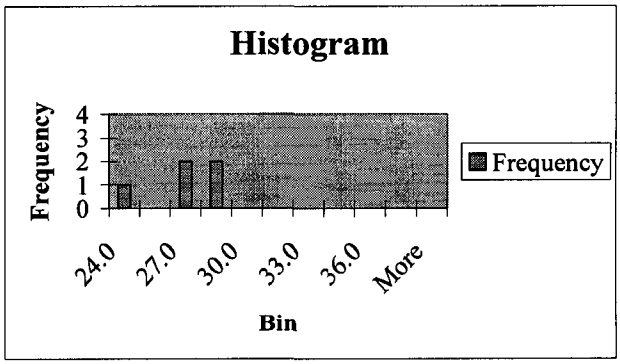
wm	9 wks	bin
M267	30.7	24.0
M269	36.3	25.5
M283	31.1	27.0
M284	27.7	28.5
M285	29.5	30.0
M298	27.9	31.5
M299	24.6	33.0
M301	28.6	34.5
M302	27.1	36.0
M304	24.5	37.5

Bin	Frequency
24.0	0
25.5	2
27.0	0
28.5	3
30.0	2
31.5	2
33.0	0
34.5	0
36.0	0
37.5	1
More	0



mm	9 wks	bin
M268	26.0	24.0
M282	26.9	25.5
M286	27.2	27.0
M287	27.9	28.5
M303	23.6	30.0
		31.5
		33.0
		34.5
		36.0
		37.5

Bin	Frequency
24.0	1
25.5	0
27.0	2
28.5	2
30.0	0
31.5	0
33.0	0
34.5	0
36.0	0
37.5	0
More	0



Variance and t-test, 12 weeks.

wf	12 wks	12 wks	mf
M264	29.5	25.6	**M265**
M266	25.2	27.4	**M281**
M279	28	27.2	**M290**
M280	30.8	23.6	**M292**
M288	27.5	23	**M296**
M289	26.5		
M291	24.1		
M293	26.1		
M297	27.5		

Descriptive Statistics

Column1		*Column2*	
Mean	27.2	Mean	25.4
Standard Error	0.7	Standard Error	0.9
Median	27.5	Median	25.6
Mode	27.5	Mode	
Standard Deviati	2.1	Standard Deviati	2.0
Sample Variance	4.3	Sample Variance	4.1
Kurtosis	-0.2	Kurtosis	-2.8
Skewness	0.3	Skewness	-0.2
Range	6.7	Range	4.4
Minimum	24.1	Minimum	23.0
Maximum	30.8	Maximum	27.4
Sum	245.2	Sum	126.8
Count	9.0	Count	5.0

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	27.2	25.4
Variance	4.3	4.1
Observations	9.0	5.0
Pooled Variance	4.2	
Hypothesized Mean	0.0	
df	12.0	
t Stat	1.6	
P(T<=t) one-tail	0.1	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.1	
t Critical two-tail	2.2	

because the ratio between the SE's is less than two, need to use a t-test: two-sample assuming equal variance

wm	12 wks	12 wks	mm
M267	35.9	29.0	**M268**
M269	43.8	29.1	**M282**
M283	37.0	30.5	**M286**
M284	31.7	31.3	**M287**
M285	32.3	27.1	**M303**
M298	33.4		
M299	27.9		
M301	35.9		
M302	32.4		
M304	27.2		

Descriptive Statistics

Column1		*Column2*	
Mean	33.8	Mean	29.4
Standard Error	1.5	Standard Error	0.7
Median	32.9	Median	29.1
Mode	35.9	Mode	
Standard Deviati	4.8	Standard Deviati	1.6
Sample Variance	22.9	Sample Variance	2.6
Kurtosis	1.2	Kurtosis	-0.1
Skewness	0.7	Skewness	-0.4
Range	16.6	Range	4.2
Minimum	27.2	Minimum	27.1
Maximum	43.8	Maximum	31.3
Sum	337.5	Sum	147.0
Count	10.0	Count	5.0

t-Test: Two-Sample Assuming Unequal Variances

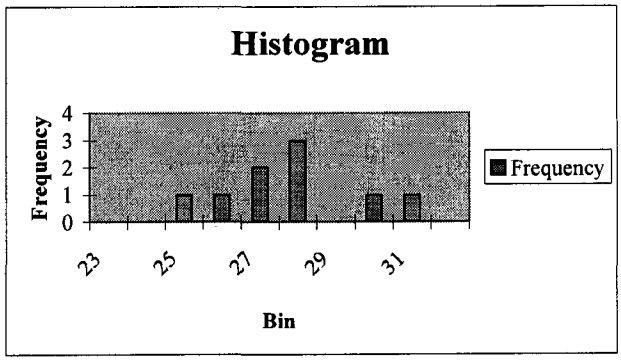
	Variable 1	*Variable 2*
Mean	33.8	29.4
Variance	22.9	2.6
Observations	10.0	5.0
Hypothesized Mean	0.0	
df	12.0	
t Stat	2.6	
P(T<=t) one-tail	0.0	
t Critical one-tail	1.8	
P(T<=t) two-tail	0.0	
t Critical two-tail	2.2	

because the ratio of the SE's is greater than two (2.10), then need to use t-test: two-sample assuming unequal variance

Histogram distribution, 12 weeks.

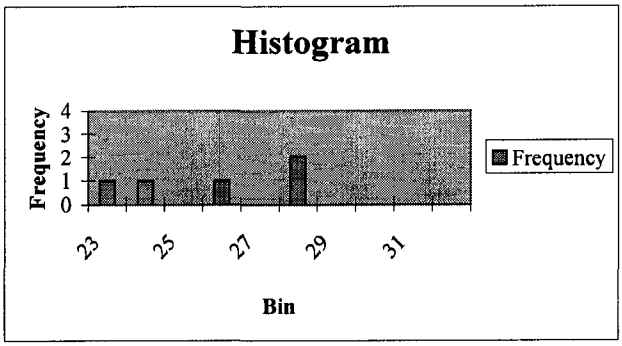
wf	12 wks	bin
M264	29.5	23
M266	25.2	24
M279	28	25
M280	30.8	26
M288	27.5	27
M289	26.5	28
M291	24.1	29
M293	26.1	30
M297	27.5	31

Bin	Frequency
23	0
24	0
25	1
26	1
27	2
28	3
29	0
30	1
31	1
More	0



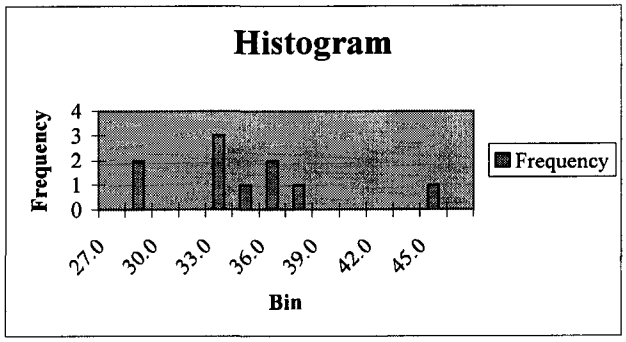
mf	12 wks	bin
M265	25.6	23
M281	27.4	24
M290	27.2	25
M292	23.6	26
M296	23	27
		28
		29
		30
		31

Bin	Frequency
23	1
24	1
25	0
26	1
27	0
28	2
29	0
30	0
31	0
More	0



wm	12 wks	bin
M267	35.9	27.0
M269	43.8	28.5
M283	37	30.0
M284	31.7	31.5
M285	32.3	33.0
M298	33.4	34.5
M299	27.9	36.0
M301	35.9	37.5
M302	32.4	39.0
M304	27.2	40.5
		42.0
		43.5
		45.0

Bin	Frequency
27.0	0
28.5	2
30.0	0
31.5	0
33.0	3
34.5	1
36.0	2
37.5	1
39.0	0
40.5	0
42.0	0
43.5	0
45.0	1
More	0



mm	12 wks	bin
M268	29	27.0
M282	29.1	28.5
M286	30.5	30.0
M287	31.3	31.5
M303	27.1	33.0
		34.5
		36.0
		37.5
		39.0
		40.5
		42.0
		43.5
		45.0

Bin	Frequency
27.0	0
28.5	1
30.0	2
31.5	2
33.0	0
34.5	0
36.0	0
37.5	0
39.0	0
40.5	0
42.0	0
43.5	0
45.0	0
More	0