Effect of Moderate Diet Restriction on Body Condition, Health, and Reproductive Performance in Female Mink (*Neovison vison*)

by

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ABSTRACT

Selection for large body size can result in the development of obesity, which in mink females is associated with poor reproduction and metabolic diseases. Caloric restriction is effective in diminishing oxidative stress and delaying aging-related diseases. This study investigated the effects of moderate diet restriction (MDR) during the fall on body condition, health, and reproductive success of mink (*Neovison vison*) breeder females. The 100 control (CTRL) females were fed according to normal farm feeding practice and the 100 sister-pair MDR females were fed about 20% less. In the fall, more ideal body weights and body condition scores (BCS) were seen in the MDR females, and they produced larger litter sizes. In addition, the MDR females exhibited less deoxyribonucleic acid (DNA) damage than the CTRL females. Telomeres were elongated in both groups. This dietary management practice is anticipated to result in significant advancement in the fur industry, both economically and welfare-wise.

LIST OF ABBREVIATIONS USED

8-oxodG	8-hydroxydeoxyguanosine
ANOVA	Analysis of variance
BCS	Body condition scores
CTRL	Control
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
IGF	Insulin-like growth factor
LH	Luteinizing hormone
MDR	Moderate Diet Restriction
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
PCR	Polymerase chain reaction
PP	Post-partum
POT1	Protection of telomeres 1
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SOD	Superoxide dismutase
TER	Telomerase ribonucleic acid
TERT	Telomerase reverse transcriptase protein
TNF-α	Tumour necrosis factor-α
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
Tris	Tris(hydroxymethyl)aminomethane

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CHAPTER 1: INTRODUCTION

The fur industry promotes intense selection for fertility and large body size as these determine profitability (Lagerkvist, 1997). In many cases, larger body size leads to the development of obese mink prior to mating. Obese females require rapid slimming prior to mating, which increases the risk of metabolic diseases such as fatty liver and nursing sickness, characterized by hyperinsulinemia and hyperglycemia and notoriously known as the largest cause of death in mink breeder females (Clausen *et al*., 1992; Wamberg *et al*., 1992; Lagerkvist *et al*., 1994). Rouvinen-Watt (2003) suggested that obesity/over-conditioning in mink breeder females is a key contributor to nursing sickness. Fatty liver disease is another syndrome associated with obesity and causes disruptions in glucose homeostasis (Bosserhoff and Hellerbrand, 2011). Fatty liver disease may also occur during the rapid period of slimming which is often necessary prior to breeding as overweight mink have poorer reproductive success than their lean counterparts (Tauson and Aldén, 1984). In fatty liver disease, fatty acids accumulate in the liver and disrupt body homeostasis (Hunter and Barker, 1996). In addition, the severity of insulin resistance is increased and extensive oxidative stress and damage may occur. Gestation and lactation exacerbate any pre-existing health issues as they cause additional weight loss from mobilization of reserves for milk production and maternal insulin resistance to ensure provision of glucose to the developing conceptus. The female, therefore, often endures a negative energy balance (NEB) during lactation (Schneider, 1996; Bell and Bauman, 1997).

Obese female mink are also likely candidates of barrenness and have increased kit losses (Tauson and Aldén, 1984). Lagerkvist *et al*. (1994) found that intense selection for

a larger body size results in smaller litter sizes, increased kit mortalities, and a decrease in pelt quality.

Glucose homeostasis and oxidative stress are also affected by body condition. Hynes and Rouvinen-Watt (2007a) confirmed a relationship between body condition and glucose regulation. Both abnormally thin and obese dams had higher glucose levels during gestation and greater variations in glucose levels throughout the reproductive cycle than dams in an ideal body condition. These disruptions in glucose homeostasis led to illness, death, or barren females in many cases. Furukawa *et al*. (2004) found an increase in the production of reactive oxygen species (ROS) in the adipose tissue of obese mice, while obese rats exhibited increased lipid peroxidation, caused by free radicals, which lead to an increase in oxidative stress (Bełtowski *et al*., 2000).

Adjusting feed availability along with body conditioning throughout the production cycle to induce a moderate and more ideal body condition and improve the health and reproductive success of mink breeder females has been suggested by Rouvinen-Watt (2003) and more recently Hynes and Rouvinen-Watt (2007a). Moderate caloric restriction from September to December may allow the female mink to maintain an ideal body condition throughout the production cycle. Nursing sickness exhibits similar characteristics to type 2 diabetes (Rouvinen-Watt, 2003) so it is hypothesized that the traditional treatment for diabetes, caloric restriction, should aid in the prevention of this illness as well as promoting the aging-retarding and anti-inflammatory benefits of diet restriction (Masoro, 1993; Masoro, 1995; Goodpaster *et al*., 1999; Barzilai and Gabriely, 2001; Dandona *et al*., 2001; Kealy *et al*., 2002). Results from numerous studies have proven the effectiveness of caloric restriction and weight loss in the management of type

2 diabetes, via improved glucose homeostasis (Fontana and Klein, 2007; Crisóstomo *et al*., 2010). Caloric restriction is also effective in diminishing oxidative stress and damage, ameliorating inflammation, delaying aging-related diseases, and increasing lifespan in several species (Kealy *et al*., 2002; Fontana and Klein, 2007; Crisóstomo *et al*., 2010). Furthermore, diet restriction has been shown to resolve hormonal and metabolic abnormalities associated with infertility and menstrual irregularity in females of reproductive age (Moran *et al*., 2003; Escobar-Morreale *et al*., 2005).

This study was conducted for two main reasons: a) to increase profitability for the mink industry; and b) to gain a better understanding of the effects of energy fluctuations on health and reproduction in mammals using mink as a model system. This project will enable a better understanding of the impacts of body condition on genomic health and reproductive performance in mink females as very little information exists about these effects in mink. It is anticipated that this research will enhance the reproductive performance of mink breeder females and will result in significant economic advancement in the fur industry. Given that mink nursing sickness and fatty liver disease are two leading causes of morbidity and mortality in adult female mink, this research is also expected to markedly improve the health and welfare of the mink.

CHAPTER 2: LITERATURE REVIEW

2.1 Mink Production and Body Weight Changes

The ranching of mink for their pelts became more of a robust industry in the 1940's (Bowness, 1996). These animals are some of the most recent to become domesticated and are only partially so (Atkinson, 1996; Murphy, 1996). A typical production season consists of breeding in March, whelping in late April to early May, weaning in June, and selection of future breeding stock in November with pelting of culled mink occurring in late November and early December (Sundqvist *et al*., 1988; Murphy, 1996; Onderka, 1996). Most ranchers primarily base their breeding selection programs on pelt quality, as well as pelt and litter size, with reductions in both of these factors leading to replacement and culling of inferior mink from the herd (Murphy, 1996). The lifespan of a mink dam is, therefore, quite short as her litter size typically decreases after the second breeding season (Largerkvist *et al*., 1993).

High reproductive performance is the most important prerequisite for a profitable mink ranch. To maximize economic profit, breeding for the largest mink is common practice as buyers desire the largest pelts possible (Lagerkvist, 1997). Producing larger mink comes at a cost and can be a challenge as this boreal year-round active species undergoes marked seasonal changes in body mass in the wild as well as in captivity (Korhonen *et al*., 1985; Korhonen, 1988; Dunstone, 1993).

Korhonen (1988) found that in eastern Finland both male and female mink body weights were highest in mid-winter, the months preceding the breeding season, and lowest in mid-summer, with a drop of 20-30%, due to changes in subcutaneous fat reserves and body water content. A later study, also in Finland, found maximum body

weights of males and females to occur in October (Korhonen and Niemelä, 1997). Feed consumption is high during late summer and autumn in order to facilitate the accumulation of energy reserves for times of food scarcity during winter as well as for thermal insulation. A decrease in feed consumption, coinciding with a decrease in body weight, occurs during the winter and spring and lasts until late summer (Tauson, 2001). Korhonen (1990) determined a relationship between feed intake and ambient air temperatures in that the colder the temperature the less the animal would consume. During summer, energy intake increases though body weight changes only to a minor extent. An increase in locomotor activity may be an explanation as activity in the mink during the spring does in fact increase (Korhonen, 1990; Korhonen and Niemelä, 1998). These variations in weight and feed intake seem to be under the control of temperature, food supply, and photoperiod (Korhonen, 1990; Korhonen and Niemelä, 1998; Tauson, 2001). By voluntarily adjusting feed intake and energy expenditure, the wild mink decreases its body weight to an appropriate level for breeding despite feeding levels.

2.2 Obesity

Mink, being seasonal breeders, have a defined breeding period with mink of the Northern hemisphere allowing male intromission beginning around March 1 (Murphy, 1996). Breeding season is a critical production point for a mink farmer as breeding results determine the success of the ranch for the rest of the year (Murphy, 1996). With today's mink ranch feeding practices, an appropriate breeding body condition, however, is not always achieved, with obesity, which can have deleterious impacts on productivity, often developing in the mink (Korhonen and Niemelä, 1998). Adipose tissue is necessary for energy regulation and the buffering of dietary fat entering the circulation (Mohamed-Ali

et al., 1998; Frayn, 2001). Lipodystrophy, the lack of adipose tissue, or obesity, the accumulation of adipose tissue, can lead to extensive changes in glucose and lipid metabolism (Frayn, 2001; Dandona *et al*., 2004). Obesity develops when there is an imbalance between energy intake and energy expenditure and is a state of inflammation as indicated by plasma concentrations of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), both inflammatory mediators which may suppress insulin signal transduction (Samartín and Chandra, 2001; Dandona *et al*., 2004). Obesity causes an increase in fatty acids in the circulation as well as an accumulation of fatty acids in glucose-metabolizing tissues such as the liver. The fatty acids entering the liver become esterified with glycerol to form triglycerides and an accumulation of triglycerides directly affects insulin sensitivity in insulin dependent tissues (Dowman *et al*., 2010).

2.2.1 Obesity and Reproductive Performance

Obesity in mink is associated with decreased reproductive performance. Clausen *et al*. (2007) reported fewer live born kits in black and brown mink females who were overweight during whelping. They also found that being overweight in November required intense restrictive feeding to reach a body condition acceptable for successful breeding. Thereafter weight was easily regained, and as a result maintaining an acceptable body condition was very challenging. In women, further consequences of obesity include birthing complications such as congenital malformations, dystocia, and other adverse pregnancy outcomes such as stillbirths, all of which compromise the health and survival of the offspring (Villamor and Cnattingius, 2006; Yogev and Catalano, 2009). Increased birthing difficulties and pup mortality rates during the perinatal period were also noted in obese rats (Rasmussen, 1998). In addition, obesity is a risk factor for

delayed onset of lactation in humans and this delay can lead to excessive weight loss in the infant, dehydration, and even death (Dewey *et al*., 2003). Not only does maternal obesity in humans increase the risk of adverse pregnancy outcomes, but modest weight gain prior to pregnancy also leads to complications during the perinatal period (Villamor and Cnattingius, 2006). This is important to consider in the mink as weight gain is a common occurrence during the production year.

Post-weaning, mink kits are fed a large quantity of high energy feed to sustain growth and yield large pelts (Atkinson, 1996). This regimen often causes the animals to become obese prior to mating (Korhonen and Niemelä, 1997). Lagerkvist *et al*. (1994) found that intense selection for a larger body size results in smaller litter sizes, increased kit mortalities, and a decrease in pelt quality. Selection for larger pelt size also encumbers litter size in the Finnish blue fox (Peura *et al*., 2007). In order to increase breeding success, the animals are therefore slimmed to an appropriate condition before mating season (Korhonen and Niemelä, 1997). Pre-mating weight loss can range from 9-25% and this rapid slimming has its consequences (Korhonen *et al*., 2002). Tauson and Aldén (1984) reported higher percentages of barren females, a decrease in the number of live born kits, and higher kits losses for females on a high feeding intensity in the fall then slimmed until late February. This slimming caused an average weight loss of 300g whereas females kept in a moderate condition during fall and winter lost less than 30g and had lower kit losses and lower numbers of barren females. Slimming has also been found to increase stereotypic behavior in mink (Damgaard *et al*., 2004).

Obesity and the associated hyperinsulinemia also affect the reproductive axis in several ways (Velloso and Schwartz, 2011). Insulin stimulates leptin production which is

known to influence ovulation, pregnancy, and lactation, and shows inhibitory effects in the gonads (Moschos *et al*., 2002). Hyperinsulinemia, accompanied by hyperleptinemia, is often seen in obese individuals and high leptin concentrations are found in serum and follicular fluid in obesity (Wabitsch *et al*., 1996; Moschos *et al*., 2002; Velloso and Schwartz, 2011). With these excessive amounts of circulating leptin, and associated increases in gonadotropin-releasing hormone (GnRH) pulse frequency, disruptions in reproduction may occur (Messinis and Milingos, 1999; Moschos *et al*., 2002). It is thought that leptin stimulates luteinizing hormone (LH) and ovarian follicle development (Moschos *et al*., 2002). Leptin receptors are present in the hypothalamus, gonadotrope cells of the anterior pituitary, granulosa, theca, and interstitial cells of the ovary. The presence of these receptors suggests a role for leptin in reproduction and it has previously been discovered that leptin accelerates GnRH pulsatility. With increasing GnRH, there is a resulting increase in LH (Moschos *et al*., 2002). Leptin may also directly stimulate LH and follicle-stimulating hormone release by the anterior pituitary by combining with its receptors on the anterior lobe (Yu *et al*., 1997). An increase in LH leads to hyperplasia of the ovarian stroma and thecal cells which further increases ovarian androgen production (Gambineri *et al*., 2002). Insulin is known to stimulate androgen production (Douglas *et al*., 2006) while hyperandrogenism leads to menstrual disturbance as it disrupts normal follicular development and disturbs ovarian function (Ramsay *et al*., 2006). The mechanism by which these disturbances occur is via the interference of androgens with estradiol-dependent signaling mechanisms and oocyte gene expression (Dumesic and Abbott, 2008). Also androgen-related is the sex hormone-binding globulin which is responsible for transporting mainly androgens and, to a lesser extent, estrogens. This

carrier protein is decreased in obese individuals resulting in an increase in free androgen and hyperandrogenism is the consequence (Pasquali, 2006). In the female mink, body weight changes are reflected in plasma concentrations of leptin and so this may be a factor in the poor reproductive performance of overweight mink (Tauson and Forsberg, 2002).

It is apparent that maternal obesity has many direct negative consequences to the dam's litter size as well as to the offspring's health and viability. Minimizing weight loss or weight fluctuations throughout the production year is, therefore, an area that warrants investigation as these factors are obviously detrimental to reproductive performance and body homeostasis. Opportunities may exist to improve industry practice in this regard.

2.2.2 Obesity and Mink Dam Health

Obesity also poses a major risk to physiological and metabolic disturbances as well as disease in the dam. Excessive adiposity in humans, and therefore excessive production and secretion of adipocytokines, can lead to a metabolic syndrome, i.e. a combination of disorders such as hypertriglyceridemia, hypertension, and hyperglycemia, and which together increase one's chances of developing diabetes and heart disease (Matsuzawa *et al*., 1999; Yaffe *et al*., 2004). In humans, the risk of type 2 diabetes, cardiovascular disease, osteoarthritis, arteriosclerosis, certain forms of cancer, dyslipidemia, joint and skeletal deterioration, and hypertension are all increased with obesity (Velloso and Schwartz, 2011; Lillycrop and Burdge, 2011; Foss and Dyrstad, 2011). Samartín and Chandra (2001) reviewed the various human and animal studies which demonstrate reduced immune system capability and increased susceptibility to disease and infection in obese individuals. Obesity is therefore also considered a risk for

the deterioration of immune function. In the future, human obesity, as a major disruptor of homeostasis, will lead to an increase in the incidence of chronic diseases (Foss and Dyrstad, 2011).

Obesity also alters hypothalamic function in such a way that energy homeostasis is disrupted and an obese phenotype becomes biologically defended (Velloso and Schwartz, 2011). Excessive adiposity leads to resistance of the hypothalamus to signals from molecules responsible for relaying energy store level information, such as leptin and insulin. This may be due to the hyperleptinemia, hyperinsulinemia, and inflammation that is associated with obesity. Increased blood glucose levels and hyperinsulinemia, characteristics indicative of insulin resistance, have, in fact been found in obese mink (Rouvinen-Watt *et al*., 2004). Hynes and Rouvinen-Watt (2007a) also confirmed a relationship between body condition and glucose regulation. Thin and obese dams had higher glucose levels during gestation and greater variations in glucose levels throughout the reproductive cycle than dams in an ideal body condition. These disruptions in glucose homeostasis led to illness, death, or barren females in many cases. Obesity is, therefore, a major challenge to glucose homeostasis. Lifespan and reproductive longevity are also affected by the level of adiposity.

In humans, longevity is greatly reduced and early mortality risk increased in obese individuals (Peeters *et al*., 2003; Finkelstein *et al*., 2010). Not only do overweight mink face the above reproductive performance risks, but are also susceptible to other metabolic disturbances, such as nursing sickness and fatty liver disease, which may arise during gestation, lactation, and weaning (Hunter and Barker, 1996; Schneider, 1996; Rouvinen-Watt, 2003).

2.2.2.1 Nursing Sickness

The body condition of the mink dam is a primary factor to consider in the prevention of nursing sickness (Rouvinen-Watt, 2003). This metabolic disorder, responsible for diminishing profits in the mink industry and holding the record as the largest single cause of mortality in lactating mink dams, mostly affects large breeder females specifically selected for their high productivity (Schneider, 1996). Other factors which increase the risk of developing nursing sickness include the age of the dam, litter size, obesity followed by extreme weight loss, and stress (oxidative and environmental) (Clausen *et al*., 1992; Rouvinen-Watt, 2003). Morbidities associated with this disease fluctuate from year to year and ranch to ranch. They can range from 0-15% while mortalities may reach as high as 8% as indicated by findings in Denmark and Canada (Clausen *et al*., 1992; Schneider *et al*., 1992; Schneider, 1996). Onset is usually seen around six weeks after parturition, typically weaning time, closely followed by mortality (Schneider and Hunter, 1992; Schneider, 1996). Nursing sickness is characterized by lethargy, loss of appetite, progressive weight loss, and emaciation. The dam may undergo rapid deterioration with symptoms such as vomiting, black-tarry feces, a staggering gait, and head tremors. She may also be reluctant to leave the nest (Clausen *et al*., 1992; Schneider, 1996). Hepatic lipidosis with distinct lipid vacuolization of hepatocytes and renal tubular epithelial cells are commonly found in females with this condition (Schneider *et al*., 1992; Clausen *et al*., 1992; Schneider, 1996).

The key triggers in the development of nursing sickness seem to be oxidative stress, non-ideal body conditions causing insulin resistance, and lactational demands (Rouvinen-Watt, 2003). These may then be followed by weaning, the most stressful event

for the dam during nursing as indicated by 2-fold increases in plasma cortisol. The dam may refuse to eat which only exacerbates the extreme weight loss problem and vulnerability to nursing sickness (Clausen *et al*., 1999; Sørensen *et al*., 2001). The commonality among the nursing sickness triggers is that they all alter glucose homeostasis (Børsting and Gade, 2000; Rouvinen-Watt, 2003).

2.2.2.2 Glucose Metabolism

As a strict carnivore species, the mink relies on diets high in protein and fat and low in carbohydrates. The mink's glucose demand is therefore satisfied by gluconeogenesis, the production of glucose from non-carbohydrate substrates such as amino acids, lactate, glycerol, and pyruvate. Mink fed diets lacking in carbohydrates can maintain glucose homeostasis by gluconeogenesis only if gluconeogenic precursors, such as amino acids, are available (Børsting and Gade, 2000).

During pregnancy, glucose is the primary nutrient for milk synthesis and for growth of the developing fetus (Bell and Bauman, 1997). The increased production of glucose and its provision to the conceptus and mammary gland is therefore vital during pregnancy and lactation. Fink and Børsting (2002) found that mink are able to synthesize large amount of glucose via gluconeogenesis. During pregnancy and lactation the dam will develop a level of maternal peripheral insulin resistance hindering glucose uptake by non-fetal tissue (Bell and Bauman, 1997). Mink farmers select for females with the largest litters, which increases the dam's glucose demand, further escalating the level of insulin resistance (Schneider, 1996).

During lactation, significant glucose synthesis by the liver is necessary as it is an important precursor for milk synthesis. Lactation is very energy demanding on the dam

(Tauson, 2001). By the 3rd-4th week of lactation, the dam produces almost 20% of her own body weight in milk each day (Wamberg and Tauson, 1998). The amount of weight loss during lactation is influenced by litter size and weight and the age of the dam with multiparous dams losing more weight than yearling dams (Hansen and Berg, 1998). Glucose synthesis is also necessary for the function of glucose-dependent tissues and organs at this time, but at this point its rate of utilization in these tissues is decreased (Fink and Børsting, 2002). Glucose turnover was found to be higher in dams with large litters as there is a need for an increase in milk synthesis and secretion with an increasing number of kits (Børsting and Damgaard, 1995). Nursing places high energetic demands on the dam. Quite often she cannot cover her losses by feed intake since the dam usually enters the lactation period in a NEB as the last part of gestation is accompanied by a decrease in feed intake (Tauson *et al*., 1998; Tauson, 2001). Weight losses can reach as high as 30% (Clausen *et al*., 1992).

During pregnancy and lactation there is a high priority to the physiological processes involved in lactation in order to maintain the litter and so these processes will proceed at the expense of other important metabolic processes which can lead to a state of disease (Bauman and Currie, 1980). In the mink's case, nursing disease may develop in certain females. It seems that some dams may develop a more severe insulin resistance, as indicated by the occurrence of hyperglycemia and hyperinsulinemia during lactation (Wamberg *et al*., 1992). Impaired glycemic control during the reproductive cycle and the extreme mobilization of body reserves in obese or severely underweight mink may be the causal factor of nursing sickness in the lactating mink (Børsting and Damgaard, 1995; Fink and Børsting, 2002; Rouvinen-Watt, 2003; Hynes *et al*., 2004).

2.2.2.3 Fatty Liver

Obesity also causes adipose tissue to become overwhelmed, which results in a loss of its buffering capacity. Fatty acids become deposited and accumulate in other tissues inducing insulin resistance in many organs and the development of fatty liver (Grill and Qvigstad, 2000). Fatty liver syndrome (hepatic lipidosis) in the mink is a metabolic disorder wherein the normal role of liver in exporting lipids as lipoproteins is inhibited by fatty acid accumulation in this organ. The liver becomes enlarged as there is accumulation of hepatic triacylglycerols. The result is fatty degeneration of hepatic cells and liver dysfunction (Hunter and Barker, 1996). Hepatic lipidosis results when mink are food-deprived and rapid mobilization of body fat occurs causing an increase in levels of triacylglycerol in the liver (Mustonen *et al*., 2005; Rouvinen-Watt *et al*., 2010). Fatty liver is a frequent finding in overweight mink due to the nutritional imbalance that comes with obesity, in dams diagnosed with nursing sickness, and in dams that undergo rapid body weight loss such as during lactation when extensive fat mobilization may be required (Wamberg *et al*., 1992; Hunter and Barker, 1996; Rouvinen-Watt *et al*., 2010). Mustonen *et al*. (2005) found that the American mink is not adapted to periods of wintertime fasting and will develop fatty livers after only 2-3 days of fasting accompanied by rapid mobilization of body fat. In similar findings by Bjornvad *et al*., (2004) mink developed fatty livers after 6-10 days of fasting. Tauson *et al*. (1998) found reduced feed intake in mink dams during late gestation. Furthermore, during late lactation inappetence may occur (Schneider, 1996). These two periods may resemble periods of short-term fasting and fatty livers are likely to result. This syndrome shows many commonalities with non-alcoholic fatty liver disease in humans and feline hepatic

lipidosis, such as occurrence mainly in individuals with excessive body fat and during metabolically stressful periods which cause disruptions in glucose homeostasis (Hunter and Barker, 1996; Brown *et al*., 2000; Bjornvad *et al*., 2004; Adams *et al*., 2005; Rouvinen-Watt *et al*., 2010). These disruptions may include decreased plasma insulin levels or insulin resistance (Adams *et al*., 2005; Shoelson *et al*., 2007; Mustonen *et al*., 2009).

2.2.2.4 Insulin Resistance

Insulin resistance, a decrease in insulin secretion and/or a reduction in insulin's ability to lower blood sugar levels, develops in obese individuals and this condition may explain what happens to overweight female mink during breeding, pregnancy, and lactation when there is either an overwhelming amount of non-esterified fatty acids (NEFA) present in the circulation or a large amount being mobilized from body tissues (Frayn, 2001; Rouvinen-Watt, 2003; Rouvinen-Watt *et al*., 2004). An accumulation of these fatty acids in glucose metabolizing tissues, as in obese individuals, leads to a decrease in glucose metabolism causing hyperglycemia. Hyperinsulinemia then occurs in an attempt to promote uptake of glucose by appropriate tissues followed by insulin resistance (Frayn, 2001). Insulin resistance may occur in obese individuals due to interference with insulin action by inflammatory mediators, such as TNF-α and IL-6, concentrations of which are elevated in insulin resistant states of obesity (Dandona *et al*., 2004).

To maximize pelt revenue, mink are bred for maximum body size. Based on the above mentioned findings this has severe consequences to the dam's health by causing disruptions in glucose homeostasis and increasing her risk of metabolic and several other

diseases. What is not known is whether restricting feed intake of mink breeder females would reduce the risk of these complications.

2.3 Reactive Oxygen Species and Oxidative Stress

The increased concentrations of free fatty acids produced in obese individuals can be toxic to the body in that they give rise to ROS which induce oxidative stress (Furukawa *et al*., 2004; Shoelson *et al*., 2007; Dowman *et al*., 2010). The level of free radicals, more specifically ROS, is increased in obese mice, which suggests an increase in oxidative stress in accumulated fat (Furukawa *et al*., 2004).

2.3.1 Reactive Oxygen Species

Free radicals are defined as molecules containing a single unpaired electron. There are two main mechanisms by which these free radicals are produced: 1) homolysis where a molecule's covalent bond is cleaved to form two molecules that each contain an unpaired electron and; 2) electron transfer where an electron is added to a molecule. Homolysis requires high energy such as ultraviolet radiation and so is less common in living bodies than electron transfer (Cheeseman and Slater, 1993). Oxygen can become a very hazardous free radical in aerobic organisms. Reduction of oxygen by addition of electrons can produce ROS which are highly chemically-reactive molecules as they possess an unpaired electron. In a biological system, the addition of a single electron to an oxygen molecule can produce a superoxide ($O_2^{\cdot -}$) which in itself is not necessarily damaging but two of these molecules can react to form the ROS hydrogen peroxide (H_2O_2). Hydrogen peroxide is an effective oxidizer and is most dangerous when transition metal ions are present as this can result in the production of the extremely reactive hydroxyl radical (OH**-**). This rapidly reacting radical, which has a very short

half-life, can damage or even destroy biomolecules such as protein, fat, and nucleic acids (Cheeseman and Slater, 1993).

Reactive oxygen species are derived from a variety of sources though most formation occurs via the electron transport chains in the endoplasmic reticulum and in the powerhouses of cells, the mitochondria. The mitochondria are the major producers of ROS and these by-products of ATP production, via oxidative phosphorylation, are thought to play a major role in aging (Yu, 1996; Miwa *et al.*, 2008). During oxidative metabolism, electron leakage occurs in the mitochondrial electron transport chain as it is not a perfectly efficient system (Beckman and Ames, 1998). One to four percent of respired oxygen becomes superoxide radicals and about 80% of these will become hydrogen peroxide molecules via dismutation reactions initiated by the enzyme superoxide dismutase (SOD). These hydrogen peroxide molecules are then at risk of being transformed into the very reactive hydroxyl radicals by the enzyme mitochondrial catalase (Yu, 1993). Turrens (1997) determined that the majority of ROS produced by the mitochondria are released at complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome-c reductase) in the electron transport chain. Oxygen radicals are also generated endogenously during peroxisomal β-oxidation of fatty acids and from the NADPH oxidase enzyme complex which is important in generating oxidants to be used by immune cells for the destruction of pathogens (Beckman and Ames, 1998; Finkel and Holbrook, 2000). As for exogenous production of radicals, ultraviolet light, inflammatory cytokines, and environmental toxins are all examples (Finkel and Holbrook, 2000).

Free radicals, at normal physiological levels, play an important physiological role in maintaining homeostasis. They have been shown to be important in growth regulation,

cell signaling, and in host defense (Finkel, 1998; Finkel and Holbrook, 2000; Forman, 2010).

2.3.2 Targets and Consequences of Reactive Oxygen Species

Proteins, lipids, and nucleic acids are the major molecules susceptible to attack by these hazardous oxidants. Lipid membranes are composed of polyunsaturated fatty acids and so are most vulnerable to attack by ROS as they react together to form a peroxyl radical. The peroxidation of lipids leads to a disruption in fluidity of the lipid membranes, eventually leading to loss of barrier function, and a potential increase in ionic permeability (Miwa *et al*., 2008).

With regard to DNA damage, oxygen free radicals are the major culprits. Reactive oxygen species can cause single and double-strand breaks in the DNA double helix, cross-linking of DNA to non-genetic materials, damage or mutations to DNA bases, replication errors, and may cause DNA to covalently bond to hazardous chemicals (Beckman and Ames, 1998; Miwa *et al*., 2008). When nucleotides become altered, genomic stability is compromised leading to premature replicative senescence, cellular dysfunction, or cell death (Chung *et al*., 1992; Griffiths *et al*., 2002; Lombard *et al*., 2005; Dalle-Donne *et al*., 2006). DNA alterations have also been linked to premature aging (Lombard *et al*., 2005). The chief example of ROS' toxicity to DNA is their reaction with DNA to form the DNA adduct 8-oxo-2'-deoxyguanosine (8-oxodG), a DNA lesion which has become a popular biomarker of oxidative stress. This oxidized form of guanine is a major concern as it can produce DNA transversion mutations (abnormal pairing) (Barzilai and Yamamoto, 2004). Accumulation of 8-oxodG with age

has been found in many species and there is much evidence linking this adduct to the pathogenesis of disease (reviewed by Takabayashi *et al*., 2004; Evans *et al*., 2004).

Mitochondrial DNA (mtDNA) is most vulnerable to damage by ROS as they are at the exact and major site of free radical production. Also, unlike nuclear DNA, mtDNA does not possess protective nucleosomes and adequate repair mechanisms, and it is partly in single stranded conformation resulting in an increase in vulnerability to damage. Oxidative damage to mtDNA can disrupt the electron transport chain and this can in turn lead to an increase in the production of oxygen radicals (Bohr and Anson, 1995; Weindruch, 1996; Finkel and Holbrook, 2000).

Damage to proteins, e.g. protein oxidation, can result in protein aggregation, which is associated with many neurodegenerative diseases and loss of enzymatic function (Cleveland and Liu, 2000; Miwa *et al*., 2008).

Lesions and alterations caused by ROS are a major health concern as they have been associated with aging, mutagenesis, and carcinogenesis (Dizdaroglu *et al*., 2002). Also of alarm is how the severity of damage to these targets affected by ROS can be exacerbated in the presence of oxidative stress.

2.3.3 Oxidative Stress

Oxidative stress is an imbalance in the production of ROS and the body's ability to detoxify or repair the damage caused by ROS through the use of antioxidants (Cheeseman and Slater, 1993). Studies have shown that increasing levels of protein in the diet increase protein oxidation rates and metabolic rates which also leads to increased oxidative stress (Fink and Børsting, 2002; Rouvinen-Watt, 2003). This finding is important to the mink industry as a typical mink diet is high in protein, especially during

breeding and lactation (Atkinson, 1996). The female mink is therefore subjected to unusually high oxidative stress if overweight during these two periods as oxidant status and levels of oxidative stress vary depending on nutritional status as well as age (Mohanty *et al*., 2002; Sharma *et al*., 2010). Studies, however, have not yet demonstrated the relationship between BMI and oxidative stress in the mink.

2.3.3.1 Obesity, Aging, and Oxidative Stress

With age, an increase in oxidative stress and damage occurs (Barja, 2004). Age is also associated with impairment of antioxidant defenses in mice, and a decrease with age in total plasma antioxidant capacity has been previously reported in humans (Rizvi *et al*., 2006; Vaanholt *et al*., 2008). An increase in antioxidant enzyme activity also occurs with age in humans (Rizvi and Maurya, 2007).

Oxidative stress is also seen in obesity. Obesity is a state of chronic inflammation which causes release of inflammatory cells, such as leukocytes (neutrophils, eosinophils, basophils, and monocytes) and monocytes from the venous system to adipose tissue. Monocytes will differentiate into macrophages, which, when activated, release cytokines such as TNF-α and IL-6, both inflammatory mediators (Coussens and Werb, 2002; Chen, 2006; Gregor and Hotamisligil, 2011). Inflammatory cells are capable of producing ROS, and so there is an increase in the production of these reactive molecules in obese individuals (Coussens and Werb, 2002; Higdon and Frei, 2003). In agreement, Mohanty *et al*. (2002) determined that fat intake increases ROS production and Furukawa *et al*. (2004) also found an increase in ROS production in the adipose tissue of obese mice. In adult male Wistar rats, it was determined that induced obesity leads to a direct increase in lipid peroxidation, caused by free radicals, and an indirect increase in oxidative stress

(Bełtowski *et al*., 2000). They hypothesize the cause to be the stimulation of mitochondrial oxidative metabolism leading to an increase in electrons leaking from the mitochondrial respiratory chain. Also of note in this study were the reduced levels of the antioxidant enzyme SOD and reduced radical scavenging ability of non-enzymatic antioxidants observed in obese rats (Bełtowski *et al*., 2000). Furukawa *et al*. (2004) found an increase in ROS production in accumulated fat and determined a close correlation between obesity and markers of systemic oxidative stress in humans.

Biological aging, otherwise known as senescence, is defined as the biological decline in an organism over time. These deteriorative changes, which occur during the adult phase, accumulate and reduce cells' abilities to withstand stress making them more susceptible to damage, disease, or loss of function. The ability to maintain homeostasis is lost and the survival ability of an organism is compromised (Masoro, 2005; Yu, 1996). Many studies suggest that, due to their harmful effect, ROS are a key component in the process of aging (Beckman and Ames, 1998). It was not until the discovery of SOD and its role in removing potentially harmful superoxide radicals that the free radical theory of aging, developed by Denham Harman in 1956, became well accepted (McCord and Fridovich, 1969; Beckman and Ames, 1998; Barja, 2004). This theory suggests that free radicals generated during normal metabolism, the majority of which are produced by the mitochondria, cause cumulative damage inside cells and the amount of damage determines lifespan (Harman, 1956). The resulting damage causes a vicious and destructive cycle as ATP production efficiency is reduced which leads to an increase in free radical output which causes further damage. Accumulation of damage can also lead to cellular dysfunction and cell senescence or apoptosis eventually leading to disease and

finally death (Weindruch, 1996; Finkel and Holbrook, 2000). In agreement with the free radical theory of aging, there exists extensive evidence of a relationship between free radicals and oxidative stress, DNA damage, genomic instability, and aging (Bohr and Anson, 1995).

Several age-associated degenerative conditions seem to be linked with oxidative stress such as cataracts, coronary heart disease, and thrombosis (Collins, 2009). Inflammation is a component of these conditions and it is known that inflammatory cells are capable of producing ROS (Coussens and Werb, 2002). Though studies have not yet made it clear as to whether oxidative stress is a cause or effect of these degenerative conditions, it certainly seems to be a recurrent key factor. Another important point when it comes to aging is that the iron content in the human body increases with age. Iron's catalytic power generates hydroxyl radical in the Fenton reaction, usually occurring to oxidize contaminants, and so an increase in iron with age means an increase in free radical production (Beckman and Ames, 1998).

The above mentioned studies illustrate that aging and obesity, both states of enhanced oxidative stress, can reduce one's antioxidant defense capacity and can drastically increase the production of ROS. These reactive molecules can ultimately cause cell death if antioxidant defenses do not succeed in eliminating them (Miwa *et al*., 2008). It is not however known whether aging and obesity exhibits the same effects in the mink.

2.3.3.2 Defense Against Oxidative Stress

There are several ways in which oxidative damage is kept to a minimum. In eukaryotes, one mode is by the body's enzymatic antioxidant defense mechanisms. Antioxidant enzymes work by either preventing the production of ROS or by scavenging and neutralizing them. Three major enzymes, SOD, catalase, and glutathione peroxidase, which are all manufactured within the body, are at play here. These enzymes protect at the cellular level (Cheeseman and Slater, 1993; Miwa *et al*., 2008).

Free radicals are substrates for SOD and the hazardous properties of free radicals are eliminated when the enzyme scavenges the radicals and neutralizes them. Superoxide dismutase accelerates the transition from superoxide to a less reactive hydrogen peroxide molecule but can also have deleterious effects as it can undergo a pro-oxidative reaction which produces radicals rather than eliminating them. The other two enzymes decompose peroxides, such as hydrogen peroxide, to form the harmless water molecule (Cheeseman and Slater, 1993; Miwa *et al*., 2008). Other non-enzymatic radical scavengers include glutathione, ascorbate, pyruvate, flavonoids, and carotenoids (Finkel and Holbrook, 2000). Our bodies also produce metal-chelating molecules, such as ferritin, which bind potentially harmful and catalytic transition metal ions (Beckman and Ames, 1998). Methods of repairing damage caused by ROS are also important in cell protection and prevent graver consequences. To prevent a buildup of damaged biomolecules, these repair systems, mostly enzymatic, eradicate them to prevent further damage to surrounding cells and tissues (Cheeseman and Slater, 1993).

The body also contains DNA repair mechanisms which help to reverse DNA lesions. The DNA damage response involves several steps. First, lesions are repaired. Base excision repair is a mechanism of cutting out lesions or replacing mismatched bases. To repair double strand breaks, this involves either non-homologous end joining, where chromosome ends are fused together or homologous recombination which uses a homologous sequence elsewhere in the genome to repair a single or double-stranded break (Karp and van der Geer, 2005; Frank-Vaillant and Marcand, 2002). Another step is the inhibition of cell-cycle progression during these repair processes and if DNA damage is too extensive, the response in this case is to induce apoptosis to prevent passing on a damaged genome (Lydall, 2009). In addition to repair mechanisms, the genome possesses structures known as the telomeres to protect itself.

2.4 Telomeres

Telomeres are G-rich long tandem hexamer DNA repeats which cap the ends of most eukaryotic chromosomes. In vertebrates they are highly conserved repeat sequences of TTAGGG at the ends of G-rich strands (Klug *et al*., 2009). They protect the chromosome ends from being recognized as double strand breaks which are potent inducers of the DNA damage response, prevent chromosomes from end to end fusion and degradation, and maintain genomic stability and integrity so that organisms can function and proliferate successfully (Houben *et al*., 2008; Lydall, 2009). Without this protective mechanism, DNA damage response pathways are activated which signals senescence, cell-cycle arrest, or apoptosis (Chan and Blackburn, 2004). These protective telomere caps, however, will eventually naturally diminish due to the end replication problem.

2.4.1 DNA End Replication Problem

During DNA replication, a short ribonucleic acid (RNA) primer binds to the 3' end of the existing single strand of DNA and replication is initiated. DNA polymerases then incorporate nucleotides to complete the synthesis of double stranded DNA. The DNA end replication problem occurs when the RNA primer is removed once replication is complete which causes a loss of about five bases of genetic material per replication and leaves a 3' overhang on the original strand since replication can only occur in the 5' to 3' direction (Verdun and Karleseder, 2007). In humans, this overhang is approximately 30-110 nucleotides in length (Hug and Lingner, 2006). Rather than remaining as a vulnerable overhang, the strand forms a loop back into the double stranded portion of the DNA. This loop, known as the t-loop, contains many telomere-capping proteins which form the shelterin complex, such as telomeric repeat-binding factor 1 (TRF1), telomeric repeat-binding factor 2 (TRF2), protection of telomeres 1 (POT1) and several other core proteins. This loop is thought to be the mechanism by which telomeres protect the ends of DNA (Karp and van der Geer, 2005; Verdun and Karlseder, 2007). This looping back of the telomere prevents chromosome end fusion and homologous recombination between telomeric regions and prevents this overhang from being recognized as damage by the DNA damage response pathway. Uncapping must, however, occur during cell replication but telomerase and other important capping proteins function to quickly reform the loop to prevent degradation, fusion, or activation of the DNA damage response (Chan and Blackburn, 2004). Several DNA-binding proteins, which bind to telomeric DNA only, have been discovered and found to be necessary for proper telomere functioning (Karp and van der Geer, 2005). Due to the

DNA end replication problem, in most cells consecutive rounds of DNA replication will result in chromosome shortening as a small portion of the telomere remains uncopied (Blackburn, 1991). The capacity of cells to replicate is finite and this limited number of cell divisions has been termed the Hayflick limit. As replication proceeds the telomeres shorten and will eventually no longer cap the chromosome ends (Zanni and Wick, 2011). The process of telomere shortening causes a dysfunction in the telomeres' role, perhaps due to the inability to form a T-loop, and results in chromosome instability. This instability can lead to chromosome fusion, changes in gene expression, and mutations (Zanni and Wick, 2011). These uncapped chromosomes are recognized as DNA damage by the cell cycle checkpoint machinery and the DNA damage machinery is activated (Ben-Porath and Weinberg, 2004). When telomeres become too short, cell proliferation ceases and the telomeres signal senescence and/or apoptosis (Calado and Young, 2009). These changes in cell proliferation are associated with aging.

2.4.2 Telomeres and Lifespan

Since telomere length generally decreases with age, it is a useful marker of the replicative history and potential of cells (Gil and Coetzer, 2004). Bize *et al*. (2009) reported that life expectancy is predicted by telomere length and attrition rate rather than chronological age, where slower attrition rates result in greater survival probabilities. Cawthon *et al*. (2003) found that in humans, a decline in telomere length contributes to mortality in several age-related diseases. A study of zebra finches by Heidinger *et al*. (2012) also revealed a relationship between telomere length and lifespan where those living the longest had long telomeres at all sampling points. Telomere length measurement is also a popular biological marker for stress and age related conditions

(Moreno-Navarrete *et al*., 2010). With time, the telomeres of various mammals and most birds will be degraded and growth arrest or apoptosis will result, unless the telomeres are preserved by the enzyme telomerase (Nakagawa *et al*., 2004; Houben *et al*., 2008).

2.4.3 Telomerase

Telomere length is maintained by telomerase, a eukaryotic ribonucleoprotein reverse transcriptase that is able to elongate existing telomeres (Blackburn, 1991; Blackburn, 2000; Karp and van der Geer, 2005). Telomerase is composed of the telomerase reverse transcriptase subunit (TERT), telomerase RNA (TER), and other telomere-associated protein subunits which all contribute to this enzymatic complex (Liu *et al*., 2004). The telomerase enzyme contains an intrinsic RNA template with a sequence complementary to that of the telomere sequence. This enzyme lengthens telomeres by adding telomeric repeats to the telomere portions of DNA, thereby synthesizing telomeric DNA (Blackburn, 2000). Telomerase therefore maintains telomere length homeostasis by counterbalancing sequence loss due to the end replication problem (Blackburn, 2000). Telomerase expression occurs in many tissues during development and differentiation but once fully differentiated, telomerase becomes absent or is expressed at very low levels in most cells. In germ cells, which are rapidly dividing, and some somatic cells, telomerase activity is retained at readily detectable levels and results in maintenance of telomeres and extension of lifespan. In most somatic cells telomerase activity is not retained and so telomeres will shorten with age (Weng, 2001; Zimmermann and Martens, 2008). During the developmental period, however, telomerase activity can be detected in most human germ and somatic cells. Telomerase was also detected in most human somatic tissues at 16-20 weeks of development yet

could not be detected in the brain. Once the neonatal period was reached, telomerase activity could no longer be detected in somatic tissues (lung, skin, muscle, adrenal, glands, and kidney) and mature germ cells (Wright *et al*., 1996).

2.4.4 Telomere Length and Attrition

Nordfjäll *et al*. (2010) determined a highly significant correlation between the child's and father's telomere length, regardless of the child's sex, and a much weaker correlation between mother and child. This shows that there is a much greater paternal influence when it comes to inherited telomere length. This correlation did, however, lessen with age of both the father and child which shows that other factors and events also influence telomere length. In a study which analyzed telomere length in white blood cells of twin pairs, telomere length was found to be highly heritable (Jeanclos *et al*., 2000). Slagboom and colleagues (1994) determined a 78% heritability for telomere length in 115 twin pairs aged 2-63 years. Along with simple genetics, telomere length is also influenced by the intrauterine environment. Stress exposure during the prenatal period has been found to influence telomere length in a negative manner and could lead to increased susceptibility to disease later in life (Entringer *et al*., 2011).

In humans, telomeres can range from 3 to 30 kilobases in length depending on the cell type (Karp and van der Geer, 2005) and, according to Slagboom and colleagues (1994), they can lose up to 31 base pairs per year. This is in agreement with findings by Chen *et al*. (2011) who found that in human leukocytes, telomeres shorten at a rate of 23-47 base pairs per year. Frenck *et al*. (1998) determined that telomere attrition in peripheral blood leukocytes is differentially regulated depending on age. Young children lose these repeats rapidly, possibly due to the high rate of proliferation that occurs during

growth and development, followed by a plateau between age four and young adulthood, and then a more gradual rate of telomere loss later in life. Nasir *et al*. (2001) determined that telomere lengths in canine peripheral blood mononuclear cells range from 12-23 kilobase pairs and telomere lengths of various somatic tissues displayed heterogeneity. A review by Nakagawa *et al*. (2004) on telomere lengths and telomere length rates of change, shows that much variation exists between species and between tissues. What is prominent in this review is the use of blood cells for telomere length determination in numerous birds and mammals with attrition occurring with time in the majority of the species sampled. Many other studies have also used these cells for telomere length determination (Frenck *et al*., 1998; Cherkas *et al*., 2008). Blood is easily obtained and life style and life stress have been previously shown to influence leukocyte telomere length with changes in length being detectable (Epel *et al*., 2004; Cherkas *et al*., 2008). Haussmann *et al*. (2003) found a relationship between rate of change in erythrocyte telomere length and lifespan in five different species of birds. The longer-lived common tern, with a maximum lifespan of 26 years and a maximum observed telomere length of about 9800 base pairs, was found to lose about 57 base pairs per year. The shorter lived zebra finch, with a maximum lifespan of 5 years and a maximum observed telomere length of about 9300 base pairs, was found to lose about 515 base pairs per year. Interestingly, the Leach's storm petrel seems to be an exception to the rule of telomere shortening with age. This bird, which can live up to 36 years, was found to have a maximum observed telomere length of about 20,000 base pairs with telomere length actually increasing with age by about 71 base pairs per year. This may be due in part to telomerase upregulation. No relationship was determined between telomere length in

early life and the animals' lifespans. In agreement with this finding, western wild mice, with a maximum lifespan of only 3.5 years, were found by Coviello-McLaughlin and Prowse (1997) to have a maximum observed telomere length of 9500 base pairs in the spleen, similar to the erythrocytes of the long-lived common tern mentioned previously. Lack of similarity between these two species with regard to lifespan may be due to the different tissues being analyzed as much heterogeneity exists in telomere length among different tissues (Coviello-McLaughlin and Prowse, 1997). Coviello-McLaughlin and Prowse (1997) also found significant gender differences in kidney, liver, spleen, and brain telomere lengths in western wild mice with females having longer mean telomere lengths than males in all cases. They also found that of the tissues sampled, regardless of gender, the brain and testis had the longest telomeres and spleen had the shortest. Since telomerase is undetectable in the brain, telomere maintenance may occur due to a mechanism known as the alternative lengthening of telomeres. Knowledge of the workings of this mechanism is sparse though recombination is thought to play a role (Henson *et al*., 2002).

In the rat it was found that a decrease in telomere length occurs with age in the liver, kidney, pancreas, and lung of males and females but this reduction does not occur in the brain (Cherif *et al*., 2003). This study also found longer telomeres in males than in females in all of the above tissues except for the brain. Studies have also found that the greatest telomere reduction rates occur in longer telomeres which may indicate a priority of the telomere length maintenance machinery to maintain and protect shorter telomeres, or perhaps longer telomeres are more vulnerable to oxidative stress (Saretzki and von Zglinicki, 2002; Nordfjäll *et al*., 2009). Telomere dynamics may also differ from one

individual to another due to differences in antioxidant defense and/or telomerase activity. Some individuals may be more resistant to oxidative stress and so less harm occurs to the telomeres leading to a longer lifespan (Bize *et al*., 2009). Telomerase activity also fluctuates throughout life and varies among tissues, leading to variations in telomere maintenance among different species and tissues (Haussman *et al*., 2007).

Heidinger *et al*. (2012) determined that reproduction, a metabolically costly event, accelerates telomere shortening. This is perhaps due to the inability to up-regulate antioxidant defense or telomere maintenance mechanisms. Epel (2009) reviewed how both psychological and metabolic stress can lead to a state of systemic inflammation and oxidative stress, the latter of which can be detrimental to the telomeres. von Zglinicki (2002) studied DNA damage induced by oxidative stress and found that the guanine regions of telomeres are especially sensitive to oxidative damage induced by ROS and this stress causes single strand breaks in the DNA. Telomeric DNA is not proficient in repairing these breaks, even less so with age (Kruk *et al*., 1995; von Zglinicki and Martin-Ruiz, 2005). An increase in oxidative stress, which increases the amount of ROS, accelerates the rate of telomere shortening. This is in agreement with findings by Cattan *et al*. (2008) who showed a decrease in telomere length in several mouse tissues after treatment to induce oxidative stress. Reactive oxygen species can modify the guanine base in DNA to form the unstable compound 8-oxodeoxyguanosine which induces DNA strand breaks (Cheeseman and Slater, 1993; Houben *et al*., 2008). A report by Valdes *et al*. (2005) found additional factors affecting telomere length: obesity, a state of heightened oxidative stress, and age.

In summary, telomere lengths have been determined in various species with much variation among and within species and among different tissues. What has been consistent in these studies, however, is the observed telomere attrition in circulating leukocytes with increasing age and stress. Telomere length has not, however, been previously documented in the mink though it is anticipated that increasing body condition, a state of increased oxidative stress and premature aging, would cause reductions in their leukocyte telomere length as shown in other mammals.

2.4.4.1 Telomere Length and Obesity

Obese women had shorter telomere lengths in their white blood cells than did lean women, and the same was found for old versus young women (Valdes *et al*., 2005). In agreement, Moreno-Navarrete *et al*. (2010) discovered a negative association between BMI and telomere length in subcutaneous adipose tissue. Al-Attas and colleagues (2010) also reported shorter telomeres in obese individuals compared to their lean counterparts, though this only occurred in males. Weight loss is one of the best known tools for the alleviation of obesity and its harmful accompanying consequences. However, with regard to telomeres, Moreno-Navarrete and colleagues (2010) determined that obese and previously obese women had shorter telomeres than individuals who had never been obese. This shows how this condition of metabolic stress, whether in the past or present, can cause irreversible telomere loss in some tissues.

Telomere shortening and its associated health and disease risks have intensified research in telomere length as a biological marker. It is suggested that telomere shortening contributes to tumorigenesis, increases risk of cancer and coronary disease, and may be a cause or perhaps a consequence of cardiovascular disease (Calado and

Young, 2009; Saliques *et al*., 2010). This measurement is, therefore, considered a good indicator of genomic and metabolic health. Since telomere length is related to oxidative stress and age of the individual, a measurement of its length is very useful for biomedical research and aging related diseases. The traditional Southern blot method of measuring telomere length, by determining a mean terminal restriction fragment length, requires a large amount of sample and therefore DNA. This method is also very time consuming and so the more rapid telomere length determination method developed by Cawthon (2009) relieved these challenges. Cawthon's method, which is appropriate for blood samples, requires only a small number of cells and gives an average of all samples cells. This assay is quite useful for observing inter-individual differences within a species (Nakagawa *et al*., 2004) and the correlation (r= 0.844) between these two telomere assays is very strong (Cawthon, 2009).

Research into telomere lengths and the factors affecting their deterioration are not yet fully developed in all species. Some of the findings are slightly obscure though what seems to be consistent is the ability to detect telomeric changes over time, whether they be attrition or elongation, in a variety of mammalian blood cells. Reinforcing its capabilities as a reliable biological marker is the recent commercialization of a telomere test which will soon be available to the public by "Telome Health" (Dickinson, 2011). The evidence of detectable changes in telomere length, with over 4400 publications currently existing related to telomere biology, aging, and disease, more than suggest it is a reliable biological marker of biological age and disease risk (Telome Health Inc., 2011).

2.5 Comet Assay

Another means of investigating genomic health is via the comet assay, also known as alkaline single cell gel electrophoresis, which has the ability to measure the consequences of metabolic stress on the deterioration of DNA. This assay was developed as a way to detect and assess single stranded DNA damage and is useful in determining genomic health status (Singh *et al*., 1988). The comet assay involves immobilizing cells in low melting point agarose then lysing cells with a detergent and high salt. The cells are then placed in an alkaline electrophoresis buffer and an electric current is applied, which causes damaged single-stranded negatively charged DNA to migrate from the nucleus towards the cathode of the system (Fairbairn *et al*., 1995). This migration of the fragmented DNA forms a tail which is observed visually after staining with SYBR Green I, which causes the 'comets' to fluoresce. The length of the tail increases with damage and the intensity of fluorescence in the tail relative to the head is a good indicator of the number of strand breaks (Fairbairn *et al*., 1995; Collins, 2004). Cells are classed from 0 (no tail) to 4 (most of the DNA in the tail) and scoring can be done visually or by computer software, though close agreement has been found between the two methods (Collins, 2004).

Oxidative and other DNA damage can be easily studied using this sensitive and rapid method (Fairbairn *et al*., 1995). Using the comet assay, Collins *et al*. (1998) found that the lymphocytes of diabetics had significantly higher values of strand breaks than controls, demonstrating the higher oxidative stress, due to elevated concentrations of lipid peroxidation products, associated with diabetes. Hynes and Rouvinen-Watt (2007b) also found the comet assay effective in determining the status of oxidative stress in mink dams

during late lactation. This method was incorporated in order to determine whether glycemic status during the reproductive cycle affects oxidative damage. During pregnancy and lactation, mink dams typically undergo maternal insulin resistance in order to support milk production and therefore the developing offspring. This response can be exacerbated if the dam is obese and experiencing excessive oxidative stress and so she may become hyperglycemic. Their study incorporated antidiabetic treatments in an attempt to prevent this exaggerated response. The comet assay determined that glycemic status did not significantly affect this measure of oxidative damage, nor did the antidiabetic treatments (Hynes and Rouvinen-Watt, 2007b)

Regardless of which assay is used to determine genomic health, it is obvious that health risks accompany short telomeres and high levels of DNA damage. Stress, psychological and physiological, is a critical factor in telomere shortening and DNA damage and thus may play a role in the longevity of female mink. Obesity, a state of oxidative stress and inflammation, has adverse health effects, causes premature cellular senescence, and may accelerate the aging process thereby decreasing life expectancy and increasing the risk of developing age-related diseases (Tzanetakou *et al*., 2012). Though it has been found previously that overconditioning in mink depresses reproductive performance, it has not yet been documented whether obesity in the mink influences oxidative stress and damage.

2.6 Caloric Restriction

Ideally, in a ranch setting the mink should have the right amount of body fat to support all periods of the production cycle, but this is rarely the case. The maintenance of a moderate body condition during the fall is recommended for female mink to avoid the

detrimental effects of excessive weight loss in the winter (Tauson and Aldén, 1984). Achieving this is quite feasible via reduced feed intake as mink are highly responsive to changes in energy supply (Tauson & Forsberg, 2002). However, the level of caloric restriction and its impact on reproductive success are important to consider as litter size in other mammals appears to be negatively affected by diet restriction. Litter size in mice, for example, was found to be reduced at 15%, 30%, and 45% dietary restriction levels (Zamiri, 1978). Reproduction and longevity are negatively correlated and so it seems that if the body invests in one, energy is directed to that single process at the cost of the other (Phelan and Rose, 2005). Longevity may be increased but perhaps at the expense of reproductive performance depending on the restriction level and resulting body condition. Low feeding intensities for yearling mink females have also caused negative effects on reproductive performance while the best reproductive performance in older females, 4 years old, was seen at lower feeding intensities (Tauson and Aldén, 1985). Female mink kept in a moderate body condition during fall and winter do not undergo excessive weight reduction prior to breeding and produce superior reproductive results as compared to females on a high feeding intensity who demonstrate impaired reproductive capacity (Tauson and Aldén, 1985). A moderate body condition created by a restriction in diet in the fall and winter resulted in larger litter sizes and less kits losses during lactation. Results by Korhonen and Niemelä (1997) show that whelping results were superior for mink females on a restricted diet and housed with a male as compared to females fed just slightly below *ad libitum*. An ideal body condition is, therefore, recommended to achieve optimum health and reproductive success.

2.6.1 Mechanisms of Caloric Restriction in Various Species

Results from many studies have demonstrated the effectiveness of caloric restriction and weight loss in the management of type 2 diabetes, which seems to share many similarities with the proposed acquired insulin resistance that precedes nursing sickness in mink. Nursing sickness seems to be a progression from a less to a more severe level of what is suggested to be insulin resistance (Rouvinen-Watt, 2003). This reduction in insulin sensitivity may be amplified in females with a less than ideal body condition (Hynes and Rouvinen-Watt, 2007a). A 25% lifetime restriction in food intake in dogs resulted in improved glucose tolerance, a delay in onset of chronic diseases, and an increase in life span (Kealy *et al*., 2002; Larson *et al*., 2003). Also, dietary restriction and weight loss in rodents and humans improves insulin sensitivity and NEFA levels, decreases ROS and oxidative damage to lipids, proteins, and amino acids, protects against oxidative damage, enhances adipose tissue metabolism, and ameliorates inflammatory cytokine levels in skeletal muscle (Masoro, 1993; Goodpaster *et al*., 1999; Barzilai and Gabriely, 2001; Dandona *et al*., 2001; American Diabetes Association, 2008; Crisóstomo *et al*., 2010).

Studies have repeatedly confirmed the benefits of energy restriction in mice and rats. In all cases the rate of aging is slowed, life span is increased, and diseases, such as diabetes, artherosclerosis, autoimmune diseases, cancer, and kidney and respiratory diseases, are prevented which decreases mortalities. This may in part be due to the decrease in production of free radicals and intensity of oxidative stress that comes with caloric restriction (Merry and Holehan, 1979; Yu *et al*., 1982; Masoro, 1995; Wanagat *et al*., 1999; Fontana and Klein, 2007). A reduction in free radical production by the

mitochondria is a proposed candidate for reduced oxidative stress as Gredilla *et al*. (2001) found that caloric restriction reduces rat heart mitochondrial production of the free radical hydrogen peroxide at complex I. A reduction in 8-oxodG was also seen in the mitochondria which may be due to the reduction in free radical production or perhaps caloric restriction improves mtDNA repair mechanisms. In addition to a decrease in ROS, which would lead to a reduction in oxidative stress and therefore tissue damage, several other metabolic adaptations may explain the mechanisms of caloric restriction's benefits. Meydani *et al*. (2011) determined that caloric restriction in humans resulted in an increase in the activity of the antioxidant enzyme glutathione peroxidase, although it did not increase the activity of other antioxidant enzymes such as SOD and catalase. This increase in one of the enzymes may partially explain reduced oxidative stress though this study found non-significant reductions in 8-epi prostaglandin F2, a lipid peroxidation product associated with oxidative stress. Caloric restriction in mice led to significant reductions of 8-oxodG, a product of DNA oxidation shown to increase with age, in several different tissues (Sohal *et al*., 1994). This demonstrates the beneficial decrease in oxidative damage associated with caloric restriction.

The reduction in oxidative damage is evidenced in a recent study by Kark *et al*. (2012) who found that lower caloric intake in men results in longer telomere length which demonstrates the benefits of a lower energy intake on this biological marker of longevity. The comet assay was also capable of revealing this reduction in oxidative damage. In a study of men and women, compared to the control group, six months of caloric restriction resulted in reduced DNA damage as measured by the comet assay (Heilbronn *et al*., 2006).

Many of the above mentioned benefits of caloric restriction are also seen in primates. For example, Colman and colleagues (2009) found that moderately restricting feed intake in rhesus monkeys led to significant increases in lifespan, slowed aging, improved insulin sensitivity and glucose homeostasis, and delayed age-associated diseases such as diabetes, cancer, and brain atrophy. A 30% restriction in caloric intake of rhesus monkeys led to a reduction in core body temperature and energy expenditure which suggests that changes in energy metabolism also play a role. These changes may be due to the body trying to conserve energy (Lane *et al*., 1996).

In mice, the expression of genes associated with metabolism and the inflammatory response are also affected by energy restriction (Matsuzaki *et al*., 2001; Dhahbi *et al*., 2004). Fontana and Klein (2007) reviewed several mechanisms by which caloric restriction may exert its benefits. The reviewed studies indicate possible up-regulation of cellular repair and survival genes and possible prevention of gene expression changes known to arise due to aging. Another proposed mechanism is epigenetic changes in gene expression via DNA methylation and histone modification (Li *et al*., 2011). With regard to the inflammatory response, Matsuzaki *et al*. (2001) found that after injecting mice with lipopolysaccharide, known for its pro-inflammatory properties, a greater suppression in the inflammatory response (i.e. production of pro-inflammatory cytokines such as IL-6 and TNF-α) was seen in energy-restricted mice versus a control group. Injury to the liver, induced by lipopolysaccharides was also diminished in the restricted mice. Furthermore, prior to and after lipopolysaccharide injection, the levels of corticosterone, which has anti-inflammatory properties, were significantly higher in the restricted mice. These results suggest a reduction in systemic

inflammation as a result of energy restriction. In agreement, old mice subjected to calorie restriction showed inflammatory cytokine levels similar to young mice and significantly lower than old and non-restricted mice (Spaulding *et al*., 1997; Avula and Fernandes, 2002). This normalization effect of caloric restriction on cytokines is important as overproduction and/or dysregulation of these cytokines can have negative consequences to health. In addition, caloric restriction in rodents has also been found to prevent lymphocyte apoptosis (Avula and Fernandes, 2002).

The anti-aging and anti-disease effects of caloric restriction may also be due to a reduction in metabolic activity, though this theory is not always supported, or a decrease in glycation as was found in skin collagen of long-term calorie restricted rhesus monkeys and in plasma proteins of calorie restricted rats (Davis *et al*., 1994; Sell *et al*., 2003; Masoro, 2005). A reduction in metabolic activity could be explained by a reduction in the thyroid hormone triiodothyronine as was found in calorie restricted rhesus monkeys (Roth *et al*., 2002).

Weight loss has also been found to alleviate irregular hormonal and metabolic characteristics that ultimately lead to infertility and menstrual irregularity. In a study by Andersen *et al*. (1995), weight loss improved insulin sensitivity in women suffering from polycystic ovary syndrome, an endocrine disorder associated with obesity, insulin resistance, hyperandrogenism, infertility, and anovulation (Gambineri *et al*., 2002; Douglas *et al*., 2006). Energy restriction in women with polycystic ovary syndrome also improved menstrual cyclicity, while hyperandrogenism was improved by weight loss induced by bariatric surgery (Moran *et al*., 2003; Escobar-Morreale *et al*., 2005). Weight

loss has also been found to prevent epigenetically induced obesity in offspring (Kral *et al*., 2006).

2.6.2 Body Conditioning in the Mink

In hopes of recruiting the widespread benefits of caloric restriction, body condition scoring will be implemented to maintain an ideal, non-obese, body condition in the female mink. A body condition scoring system, with scores ranging from 1-5, was developed by Rouvinen-Watt and Armstrong to score mink (Hynes *et al*., 2004). Clausen (2006) recommends using this system to evaluate and select females in good condition for breeding as it is a rapid and more reliable method of gauging the female's condition than weighing given that it indicates whether a female is overweight or simply big. This research project will attempt to restrict the female's diet during the fall when longitudinal growth is complete in such a way that an ideal body condition is held throughout the production year rather than undergoing extreme weight fluctuations (Lagervkvist *et al*., 1993). Maintaining this ideal body condition should therefore reduce the ill health effects of being overweight such as increased oxidative stress. Diet restriction in mink dams during the fall is expected to reduce their metabolic stress in such a way that DNA damage and the rate of telomere shortening should be reduced during the production cycle. It is hoped that this reduction in stress will consequently lead to improved health and an increase in reproductive performance. The two incorporated assays will therefore provide accurate and useful information on the health and integrity of the mink dam's DNA and will help in the assessment of her reproductive longevity.

CHAPTER 3: OBJECTIVES AND HYPOTHESES

The objectives of the proposed research are:

1. To study the effects of a MDR from September to December on the body condition, health, and reproductive performance of mink breeder females.
2. To examine the effects of a MDR on genomic health as measured by molecular genetic markers of oxidative stress and aging in mink breeder females.

It is hypothesized that a MDR during the fall in mink breeder females will lead to improved reproductive performance via an increase in the number of offspring produced. Improvements in genomic health and integrity are also expected.

CHAPTER 4: MATERIALS AND METHODS

4.2.1 Animals

This project consisted of the first two years of a five-year research program on female mink reproductive longevity. The first year of the trial began in September, 2009 with the second year beginning in September, 2010. A total of 200 standard black female mink, selected based on standard ranch criteria for breeder females and housed individually in standard sized cages in a multi-row barn at the Canadian Centre for Fur Animal Research (Truro, Nova Scotia) were included in the trial. The diet consisted of daily standard wet mink feed which was commercially produced. Water was available *ad libitum* from a nipple drinker. Animal husbandry conditions and experimental procedures were all in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993) and approved by the Animal Care and Use Committee of the Nova Scotia Agricultural College, Truro, Nova Scotia (ACUC File# 2009-037 and 2010-049).

4.2.2 Treatments

One-hundred females were placed in the CTRL group and fed according to normal farm feeding practice (Rouvinen-Watt *et al*., 2005), in which the dietary allowance was adjusted daily, based on the quantity of feed not consumed by the morning of the following day. The other 100 full-sister females were placed in the MDR group and fed ~20% less than the CTRL group from September to December in order to maintain an ideal body condition throughout the production year (Rouvinen-Watt *et al*., 2005). For feed analysis report values see Appendix 1. Activity level and weather conditions were taken into account and so the amount of restriction varied, but did not

exceed 20%. The mink were checked for general health status twice daily and all dam mortalities were sent for post-mortem examination at the NSDA Veterinary Services Pathology Laboratory, Truro, Nova Scotia. The incidence of reproductive problems such as dystocia, stillborn kits, mastitis, nursing sickness, and fatty liver disease were recorded. The females were followed up for two reproduction cycles. The experimental design and entry and exit points are illustrated in Figure 1.

To maintain a group of 200 females per year throughout the study, eliminated pairs were replaced with new yearling sister pairs in September with one sister randomly placed in each group. New pairs were selected based on standard ranch criteria for the selection of new breeder females. A pair was eliminated in September if she and/or her sister died, became ill, or had not weaned one or more kits. Industry standard is to keep a female who has weaned at least three kits but as this is a five year reproductive longevity study, in order to obtain a larger sample size of older females with each production year, the criteria were made more accommodating.

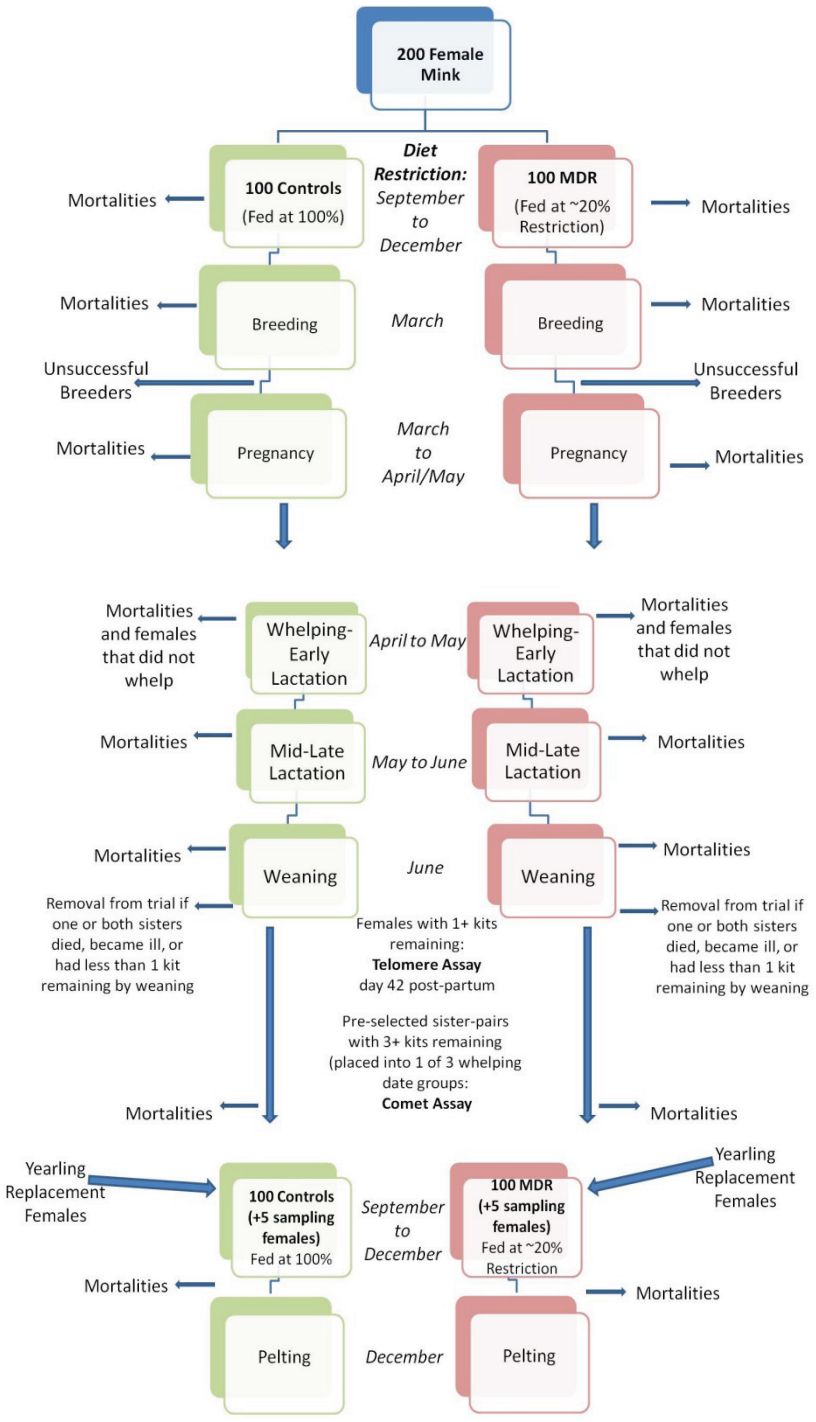

Figure 1. Project stages throughout one full production year for trial mink dams.

4.2.3 Measurements

Body condition scores and body weights measured to the nearest gram were recorded on a monthly basis except in March, when breeding took place, and during lactation where weights were recorded 1 day post-partum (pp) and every three weeks after that until weaning when the kits were six weeks of age (Rouvinen-Watt *et al*., 2005). The body condition scoring system developed by Rouvinen-Watt and Armstrong was used to score the dams where a score of 1 is very thin, 3 is ideal, and 5 is obese (Hynes *et al*., 2004). In order to support normal growth and behaviour patterns, females were housed with their male littermates until October and individually after this time.

During breeding in March, full-sister pairs were bred to the same male. The number of mating attempts as well as the occurrence of single or double matings was recorded. During whelping, dam BCS and weights, sex and number of live and dead kits, and group weights, male and female, were recorded on day one and twenty-one. All full-sister pairs where one or both females did not whelp,had no kits remaining by weaning, or were ill were eliminated from the trial.

At day forty-two post-partum, time of weaning, individual kit weights were recorded and the dam was again weighed, body condition scored and moved to a weaning pen.

4.2.4 Blood Sampling

4.2.4.1 Telomere Assay

To determine the integrity of the females' DNA using the telomere assay, blood samples by toenail clipping were collected onto a Whatman® FTA Classic card, which entraps the DNA in a matrix, at the beginning of the trial in September and on day forty-two post-partum. For the 2009 baselines, blood was collected onto

Whatman® FTA Bloodstain cards. It was later determined that the blood stain cards are intended for short term sample storage and the Whatman® FTA Classic cards for longer term storage. In order to minimize handling stress, if a dam was part of the twenty-four full-sister pairs pre-chosen for the comet assay analysis, she was sampled for the telomere assay on the pre-scheduled comet assay sampling day. Sister-pairs who did not have at least one kit remaining by weaning were eliminated from telomere assay analysis. The criteria was originally 3 or more kits by weaning though in order to obtain a larger sample size, the criteria was reduced to only 1 or more kits by weaning.

4.2.4.2 Comet Assay

Baseline sampling of 24 preselected sister pairs took place when the trial began in September 2009 and when new yearling females joined the trial in September 2010. Sampling also took place at weaning (day 42 pp). Females that began in the trial in September 2009 had two weaning samples taken over the span of this research while females that began in the trial in September 2010 had only one weaning sample taken. At weaning, the criteria to continue in the trial was sister pairs which each weaned at least three live kits as this is the typical industry standard for keeping a breeder female for the next mating season. If a sister pair sampled for the comet assay in September did not meet this criterion, a replacement pair of similar baseline weight and body condition score was sought. Each animal was assigned a comet assay code number, and if eliminated, the replacement pair was given the comet assay codes matching the eliminated pair. Blood samples were collected into a single Fisher Scientific heparinized microhematocrit tube (Thermo Fisher Scientific, Delaware, USA) per female by toenail clipping and placed on ice protected from light.

4.2.5 Laboratory Analyses

4.2.5.1 Telomere Assay

4.2.5.1.1 DNA Extraction- Samples

To extract DNA from the blood samples collected onto the Whatman™ FTA™ cards, a punch was taken from the card and placed into a 1.5mL microcentrifuge tube. The captured nucleic acids were then purified according to the Whatman™ FTA™ nucleic acid purification protocol with slight modifications (Whatman™, Kent, United Kingdom). A 1.2mm Harris micropunch was used to remove a core from the FTA card which was placed on top of a cutting mat sterilized with 70% ethanol. The micro punch was sterilized by punching a core from an FTA card soaked with a solution of TE Buffer, consisting of 1M Tris(hydroxymethyl)aminomethane (Tris) and 0.5M ethylenediaminetetraacetic acid (EDTA), pH 8.0, then punching a core from a 70% ethanol soaked FTA card, and lastly the TE buffer soaked card. This sterilization step was repeated in between punching of all samples to prevent contamination from sample to sample. Each core was washed with 200 µL of Whatman® FTA purification reagent and incubated for five minutes at room temperature. The FTA purification reagent was drained from each microcentrifuge tube and discarded and the washing step repeated. Next, 200 µL of TE buffer was added to each tube, mixed by vortexing, and the samples were incubated for three minutes at room temperature. The samples were centrifuged at 21,130 x g for fifteen seconds and the liquid drained and discarded. Fifty µL of nuclease-free water (QIAGEN, Hilden, Germany) was added to each microcentrifuge tube and the tubes were incubated at 95°C for twenty minutes. The liquid portion was removed and transferred to a new sterile microcentrifuge tube. Samples were

initially stored at 4°C until all samples from that sampling period were purified, which was typically 1-2 days.

A few samples were initially selected to evaluate the purity of the DNA, based on the ratio of absorbance readings at 260 and 280 nm, and to obtain the concentration using the Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). Sample readings were found to be too low for accurate nucleic acid concentration determination. Gel electrophoresis, using a 1% agarose gel, was performed to evaluate the DNA quality and the gels showed little to no DNA present. A polymerase chain reaction (PCR) was performed on a few random samples with the telomere primers shown in Table 1 and electrophoresis performed once again. In this case bands were visible which confirmed the presence of DNA.

Once a full sample set for a certain time period (baseline or weaning) was extracted, samples, ready for use in a real-time polymerase chain reaction (RT-PCR) using the LightCycler® 480 II (Roche Diagnostics GmbH, Rotkreuz, Switzerland) were transferred to a 96-well Progene PCR® microplate and the plate was sealed with a MicroAmp™ clear adhesive film and stored at -80°C for subsequent analysis. A few weeks prior to running the samples in the assay, samples obtained from sister pairs meeting the weaning specific criteria were transferred from the storage plate to a 96-well loading plate for ease of transferring samples to a 384-well plate via the epMotion 5075 liquid handling system (Eppendorf, AG, Hamburg). Storage plates were then stored at -80°C until used in RT-PCR analysis.

For the 2009-2010 baseline and weaning periods, 51 pairs meeting the criteria remained and were included for telomere length analysis. For the new yearling females

that began in the trial in September of 2010, 31 of those pairs met the assay criteria at weaning while 20 multiparous pairs met the assay criteria at this time.

4.2.5.1.2 DNA Extraction- Standard

In order to obtain a relative measure of telomere length for the collected samples, serial dilutions of mink genomic DNA of known concentrations, extracted from liver, were used as a standard. Deoxyribonucleic acid from mink liver tissue was extracted using a modified version of the AutoGenprep 245T DNA extraction from tissue procedure (AutoGen, Inc., Holliston, Massachusetts) which started with grinding of the liver to a powder under liquid nitrogen using a mortar and pestle. Next, about 2 g of powder was mixed with 5 mL of AutoGen tissue lysis buffer with proteinase K (2 mg/mL). This mix was incubated overnight for approximately 18 hours at 50°C. Ribonuclease was then added to a final concentration of 0.1 mg/mL and incubated at 50°C for one hour. Next, 2.5 mL of water and 7.5 mL of AutoGen deproteinization buffer were added and this was mixed and centrifuged at 3000 g for 30 minutes. The supernatant was transferred to a fresh tube where an equal volume of isopropanol was added and mixed gently. The DNA was spooled (Sambrook and Russell, 2001), transferred to a fresh tube and washed with 70% ethanol. The DNA was dried briefly in a Savant SpeedVac® SC110 apparatus (Thermo Fisher Scientific, Delaware, USA) and resuspended in 2.5 mL of 10 mM Tris, pH 8.0. For a final purification, the DNA was re-precipitated using sodium acetate and 100% ethanol, centrifuged for 20 minutes at 3000 g, washed with 70% ethanol, dried briefly in a Speedvac® (Thermo Fisher Scientific, Delaware, USA) and the pellet was resuspended in 5 mL of 10mM Tris pH 8.0. The final concentration, obtained with the Nanodrop ND1000 spectrophotometer (Thermo Fisher

Scientific, Delaware, USA), was 200 ng/uL. The Nanodrop ratios of the A260/A280 and A260/A230 readings were used to assess purity. The quality of the DNA was determined by 1% agarose gel electrophoresis. The DNA was subdivided into 50 μL aliquots in microcentrifuge tubes and frozen at -80°C.

4.2.5.1.3 Reference Gene

In order to account for sources of variation, such as pipetting, the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizing control. The housekeeping gene GAPDH has been previously used as a normalizing control in telomere length and telomere gene expression studies (Lin *et al*., 2006; Bize *et al*., 2009). Telomere RT-PCR and GAPDH RT-PCR were performed in separate 96-well plates.

4.2.5.1.4 Primers

The amplicon for the telomere sequence consists of the hexamer TTAGGG repeat which can be repeated many thousands of times. To target this area of repeated sequence, Cawthon (2009) designed primers to specifically amplify these telomeric repeats. The two primers, *telg* and *telc*, generate a short, fixed-length product. During the first cycle, *telg* is able to prime DNA synthesis on the DNA sample possessing the telomeric sequence. *Telc* is unable to do so as it possesses a mismatched base at its 3' terminus. At this point, *telg* and *telc* are also unable to bind to each other to form primer-dimer. During the second cycle, *telc* can anneal to the extension product of the *telg* primer and this hybridization is what allows for the synthesis of a product of discrete length.

Table 1. Forward and reverse primers used for RT-PCR for generating mink-specific telomere and GAPDH sequences.

Target	Primer	Primer Sequence 5'-3'	Amplicon (bp)
Telomere	*telg*	ACACTAAGGTTTGGGTTTGG GTTTGGGTTTGGGTTAGTGT	79
	telc	TGTTAGGTATCCCTATCCCTA TCCCTATCCCTATCCCTAACA	
GAPDH	*Forward*	TGACAAAGTGGT CATTGAGAGCAA	177
	Reverse	AGAAAGCTGCCAAATACGATGACA	

4.2.5.1.5 Reaction Conditions and Optimization

For a 10 µl reaction, the RT-PCR mix was composed of: 1µL of undiluted sample complementary DNA of unknown concentration, 1X SsoFast^{TM} EvaGreen® Supermix (Bio-Rad, Hercules, California), and 600 nM of each forward and reverse primer, in nuclease-free water (QIAGEN, Hilden, Germany).

The thermal cycling conditions for the telomere assay were as follows: enzyme activation at 98°C for two minutes, two cycles of 98°C for five seconds and 49°C for twenty seconds for primer conversion, forty cycles of 98°C for five seconds for denaturation, and 62°C (single acquisition) for 15 seconds for annealing and extension. To produce a melt curve, incubation at 98°C for ten seconds occurred, followed by incubation at 55°C for one minute and finally a ramp in temperature to reach 95°C with five acquisitions per °C. The plate was then cooled at 40°C for thirty seconds. These conditions were adapted from Cawthon's (2009) and optimized for the Ssofast PCR reaction mixture. The thermal cycling conditions for the GAPDH assay, based on Bio-Rad's recommended cycling conditions, were as follows: enzyme activation at 98°C for

two minutes, forty cycles of 98°C for ten seconds for denaturation, and 55°C (single acquisition) for 20 seconds for annealing and extension. To produce a melt curve, incubation at at 95°C for ten seconds occurred, followed by incubation at 55°C for one minute and finally an increase in temperature to reach 95°C with five acquisitions per °C.

Prior to using samples and standards in a quantitative real-time polymerase chain reaction (qRT-PCR), optimization steps were required. The mink DNA standard was used to prepare 3-fold serial dilutions in order to generate an appropriate standard curve for each assay for relative quantification of the samples. Deoxyribonucleic acid standard stocks were diluted 2-fold for the telomere assay and 20-fold for the GAPDH reaction using 10 mM Tris pH 8.0 just prior to setting up the RT-PCR runs. The concentration of the first standard for the telomere assay was 100 ng/uL and 10 ng/uL for the GAPDH reaction. All samples and standards were assayed in triplicate and a full standard curve was repeated on each 384-well plate.

4.2.5.1.6 Telomere Length Determination

The ratios of telomere concentration to GAPDH concentration were used to determine a telomere length relative to that of the mink DNA reference standard. These ratios were then compared between the CTRL and MDR groups.

4.2.5.2 Comet Assay

The Trevigen® higher throughput comet assay (single cell gel electrophoresis assay) system and reagents were used to detect and contrast the levels of DNA damage in the CTRL and MDR females. The alkaline (pH >13) comet assay was performed according to the manufacturer's protocol with minor modifications (Trevigen

Inc., Gaithersburg, MD). The alkaline comet assay was used, as opposed to neutral, as it is a more sensitive approach capable of detecting both single and double stranded breaks (Singh *et al*., 1988).

In dim light, 50 µL of blood was pipetted from the heparinized microhematocrit tubes and added to 1mL Invitrogen™ RPMI-1640 media (Life Technologies Corporation, Burlington, Ontario) in a microcentrifuge tube. The RPMI-1640 is a medium used to keep mammalian cells viable and contains the reducing agent glutathione along with high concentrations of vitamins such as biotin and vitamin B_{12}. The blood and RPMI-1640 mixture was then gently mixed and centrifuged at 1500 RPM for five minutes. The supernatant was removed and 600 µL of cold, Ca^{2+} and Mg^{2+} free, 1X phosphate buffered saline (137 mM MaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) was added to wash the cells. Samples were again mixed and centrifuged at 1500 RPM for five minutes. The supernatant was removed and cells resuspended. Five microlitres of each sample's cells were pipetted and mixed into 300 µL of molten (37°C) low melting agarose. Optimization steps determined that 5 µL of blood produced sufficient amounts of cells. Thirty µL was pipetted and quickly spread onto sample areas of the 20-well warmed slides in duplicate. Slides were chilled in the dark in a refrigerator for 30 minutes to accelerate gelling of the agarose and placed in chilled lysis solution (Trevigen® lysis solution, dimethyl sulfoxide) for one hour in the refrigerator to allow for full lysis of leukocytes. Following this, the slides were then placed in an alkaline unwinding solution (200 mM NaOH, 1mM EDTA, pH >13) for one hour at room temperature in the dark to allow for leukocyte DNA unwinding. The Trevigen® electrophoresis tank was filled with prechilled alkaline electrophoresis solution (200 mM NaOH, 1 mM EDTA, pH >13) and

the slides placed into the slide tray and immersed in the electrophoresis solution. The cells were electrophoresed in the dark for 30 minutes at 21V (1Volt/cm). Slides were neutralized twice in distilled water, 5 minutes each, and once in 70% ethanol for 5 minutes and fully dried at room temperature. Just prior to scoring, each slide well was stained with 50 µL of diluted Trevigen® SYBR® Green 1 (10,000X concentrate in DMSO in TE buffer, pH 7.5) (Trevigen Inc., Gaithersburg, MD) and refrigerated for 5 minutes. Excess stain was removed and the slide dried completely, protected from light, at room temperature.

A Leica DMLB fluorescent microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) with a blue filter attached to the Nikon Coolpix 4500 digital camera (Nikon Corporation) was used to view and record fluorescing cells at 40X. The captured photos were later analyzed in a double-blind test using Image-Pro Express v. 4.5 Image Analysis Software (Media Cybernetics, Inc., Bethesda, MD). Cells were scored from 0 to 4 (Figure 2) based on the extent of DNA damage present where zero represents intact and undamaged DNA with no tail and 4 represents extensive DNA damage where most of the DNA is in the tail (Collins, 2004). The software was used to aid in counting and tracking scored cells by highlighting scored cells with four different symbols representing each damage level score. For each female, five images were captured with varying amounts of cells per image. The location of these five images were selected in order to have a good representation of the top, bottom, center, left, and right of each well. Twenty cells were scored visually beginning from the left center of the images and moving to the right. This region was selected and used for the scoring of each image in order to be consistent from image to image. The total score of each female was between 0 and 400.

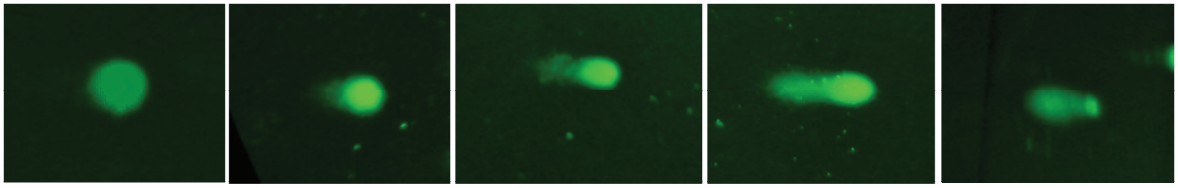

Figure 2. Depiction of comet assay cell scoring system. Scores range from 0 (no damage) to 4 (most of the DNA in the tail) (left to right).

4.2.6 Statistical Analysis

The experimental design was a randomized block design with pair as a blocking factor. The responses: body weight, body condition score, body weight loss during lactation, conditioning, litter size, and genomic health data were analyzed using the Proc Mixed Analysis of Variance (ANOVA) procedure in SAS® v.9.2 (SAS Institute Inc., Cary, NC, USA) to examine the effects of the factors (group, years in study, time). The repeated measures function was incorporated into the model when the measurement of the dependent variable was repeated such as for the multiparous comet scores and telomere lengths as well as the body weight and BCS.

For categorical responses, the CATMOD procedure in SAS® v.9.2 (SAS Institute Inc., Cary, NC, USA) was used to determine the effects of the factors. For categorical responses where only one factor was of interest (group), the chi-square goodness of fit test was performed in Minitab®16 (Minitab Inc., State College, PA, USA). Correlation and regression analyses were also performed using Minitab®16 (Minitab Inc., State College, PA, USA). Statistical significance was set at $P<0.05$ for all statistical tests. The validity of assumptions required for the tests were verified and if violated, appropriate actions, such as transformation, were taken (Montgomery, 2005).

CHAPTER 5: RESULTS

5.1 Body Weights

For body weights, when all of the females were included in the analysis, the time, years in study, and diet 3-way interaction effect was found to be significant ($P<0.001$). The primiparous and multiparous MDR females did maintain significantly lower body weights than their CTRL sisters during the fall and winter ($P<0.05$), except for the multiparous females in February ($P=0.377$) (Figure 3). The primiparous MDR females had significantly lower body weights than the CTRL females for the remainder of the production year ($P<0.05$) except for at day 21 of lactation and August. The multiparous MDR females did not differ in body weights from the CTRL females from February to September. Prior to mating (January-February), the primiparous MDR females had to gain significant amounts of body weight while the CTRL females had to lose weight. As for the multiparous females, at this point the MDR females did not gain body weight while the CTRL lost weight.

During gestation, March to April, although body weights were not measured in March due to the risk of causing embryo loss (Hunter and Schneider, 1996), all dams gained weight. From April to whelping (day 1 pp) all females lost weight as expected. From day 1-21 pp, both the primiparous and multiparous MDR females did not lose significant amounts of weight while the CTRL dams did. From day 21-42 pp, all females lost weight ($P<0.001$). From day 42 pp to July all females of both parity groups, except for the multiparous MDR females, lost significant amounts of body weight ($P<0.01$). From July to August, all females were able to regain their weight post-lactation except for the multiparous CTRL females.

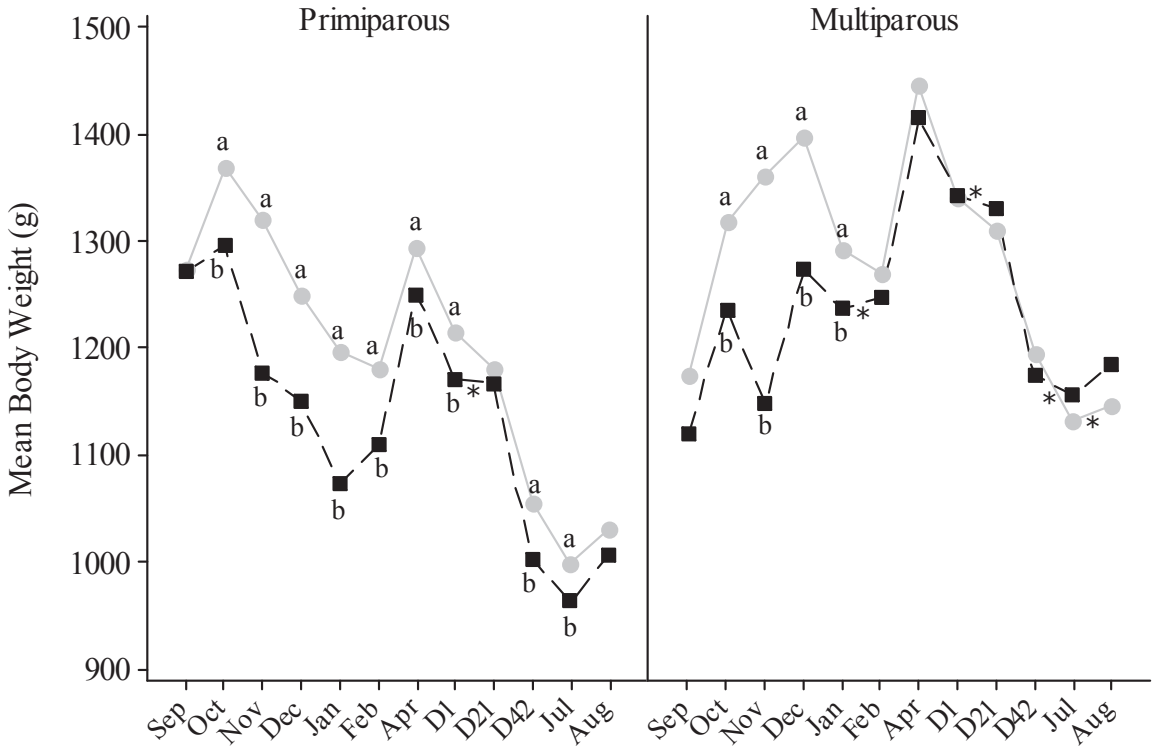

Figure 3. Mean body weights of CTRL (•) and MDR (■) female mink (D1= day 1 post-partum (pp), D21= day 21 pp, D42=day 42 pp). a-b indicates significant differences ($P<0.05$) between groups within time points while absence of letters indicates no significant differences between groups within time points. * indicates no significant change within a group from one time point to another. Primiparous females n=157 in each group and multiparous females n=43 in each group. Due to statistical analysis restrictions, differences between the groups at the baseline measurement (September) and changes from baseline to October could not be determined.

5.2 Body Condition Scores

In the months of October, November, February, July, August, and on day 21 pp both parity groups had BCS that were not different from each other (Figure 4). All other time points showed significant differences in BCS between the groups with the primiparous females having lower scores ($P<0.05$). The primiparous females had reduced BCS from October to November while the BCS of the multiparous females did not change. From November to December the multiparous females again did not change while the primiparous females reduced their condition from November until January to just below the ideal BCS of 3. The multiparous females were effectively conditioned to reach the ideal BCS of 3 from December to January. From January to February, neither parity group changed in terms of BCS. Both groups increased their mean scores from February to April. From April to day 1 pp, the primiparous females had reduced scores while the multiparous females did not. From day 1 pp to day 42 pp, both groups lost body condition. From day 42 pp to July and from July to August, the primiparous females increased their BCS while the multiparous females' scores did not change during these two time periods.

The implemented regime of dietary restriction was successful in maintaining lower and more ideal BCS in the MDR females during the fall, winter, and spring ($P<0.01$) (Figure 5). From October to November the CTRL females increased their BCS significantly while the BCS of the MDR females were reduced to reach the ideal score of 3. From November to January, the BCS of the CTRL females had to be reduced in order to achieve a condition suitable for optimal breeding success. From January to February, just prior to breeding, the CTRL females were maintained at this ideal body condition.

The MDR females, however, maintained their ideal BCS from November to December when their BCS were reduced slightly below the ideal body condition score of 3. This body condition was maintained until prior to breeding. Both groups had significant increases in BCS during gestation ($P<0.001$), whereas during lactation, the scores decreased in both groups ($P<0.01$). From day 42 pp to July, neither group increased their mean BCS. However, from July to August, the MDR females significantly increased their BCS ($P<0.001$) while the CTRL females did not.

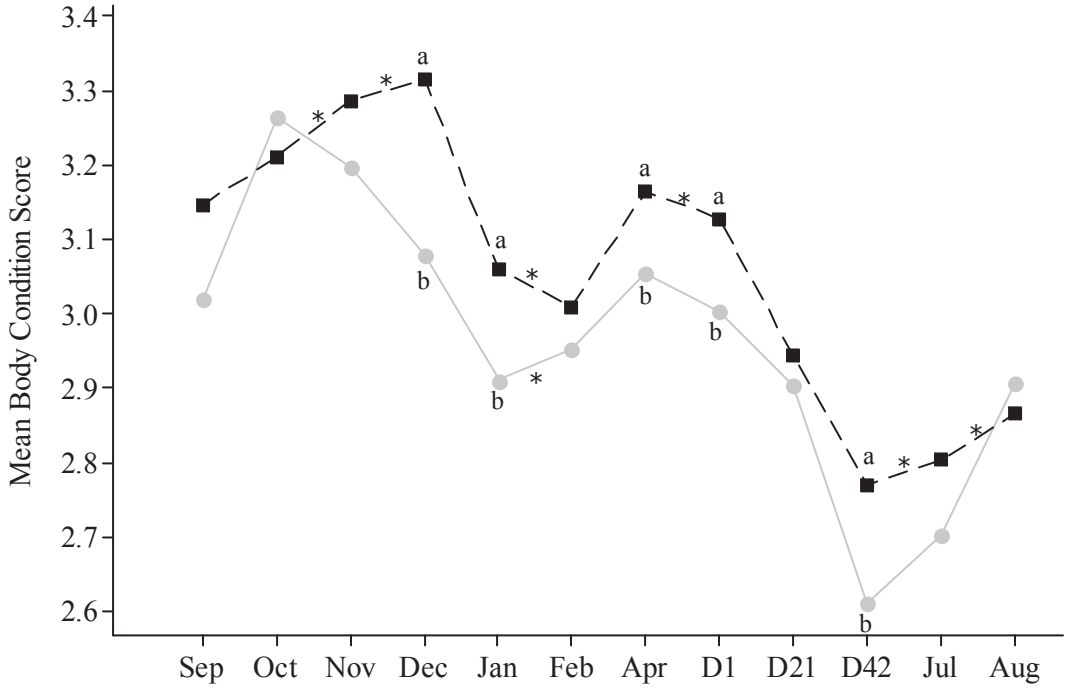

Figure 4. Mean body condition scores of primiparous (•) and multiparous (▪) female mink (D1= day 1 post-partum (pp), D21= day 21 pp, D42=day 42 pp). a-b indicates significant differences ($P<0.05$) between parities within time points while absence of letters indicates no significant differences between parities within time points. * indicates no significant change within parity group from one time point to another. Primiparous females n=314 and multiparous females n=86. Due to statistical analysis restrictions, differences between the groups at the baseline measurement (September) and changes from baseline to October could not be determined.

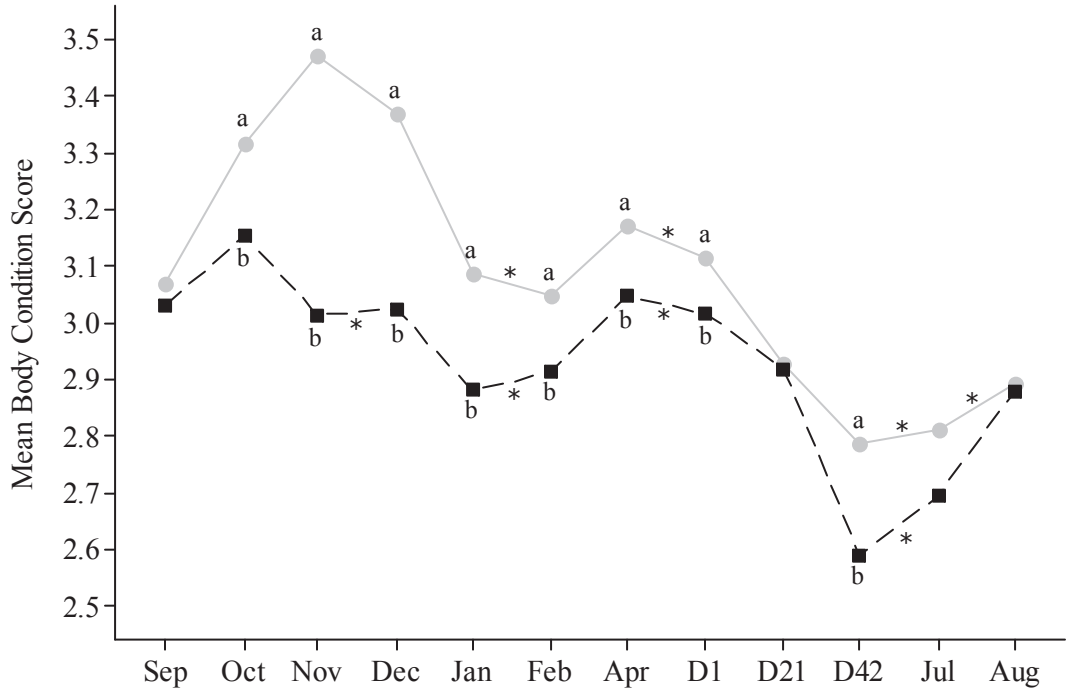

Figure 5. Mean body condition scores of CTRL (●) and MDR (■) female mink (D1= day 1 post-partum (pp), D21= day 21 pp, D42=day 42 pp). a-b indicates significant differences (P<0.05) between groups within time points while absence of letters indicates no significant differences between groups within time points. * indicates no significant change within a group from one time point to another. CTRL females n=200 and MDR females n=200. Due to statistical analysis restrictions, differences between the groups at the baseline measurement (September) and changes from baseline to October could not be determined.

5.3 Body Weight and Body Condition Scores

The body weights of all dams of all time points (n=3952) were significantly correlated with BCS (r=0.656, P<0.001). Regression analysis showed a weak regression with an R^2 of 0.43 which shows that only 43% of the variation in BCS was explained by body weight.

5.4 Conditioning

During the fall, the primiparous and multiparous CTRL females gained significantly more (9.53%±0.61 and 12.15%±1.19) (P<0.001) body weight than the MDR females (3.88%±0.61 and 4.37%±1.17) (Figure 6). As for winter percent body weight loss, the primiparous and multiparous CTRL values were 13.09%±0.61 and 10.29%±1.23 and the primiparous and multiparous MDR values were 13.52%±0.61 and 2.33%±1.23. The winter conditioning for breeding resulted in weight losses that were not significantly different (P=0.613) between the groups for yearling females but were (P<0.001) for the two year old females where the multiparous CTRL females had to lose significantly more body weight during the winter than their MDR sisters in order to achieve a body condition suitable for breeding success.

The two year old CTRL females had a tendency to gain more weight (P=0.050) in the fall than the yearling females and the multiparous CTRL females lost significantly less (P=0.043) percent body weight during the winter than the yearling females. No parity differences existed for fall percent weight gain of the MDR females but winter weight loss was significantly less (P<0.001) in the two year old MDR females.

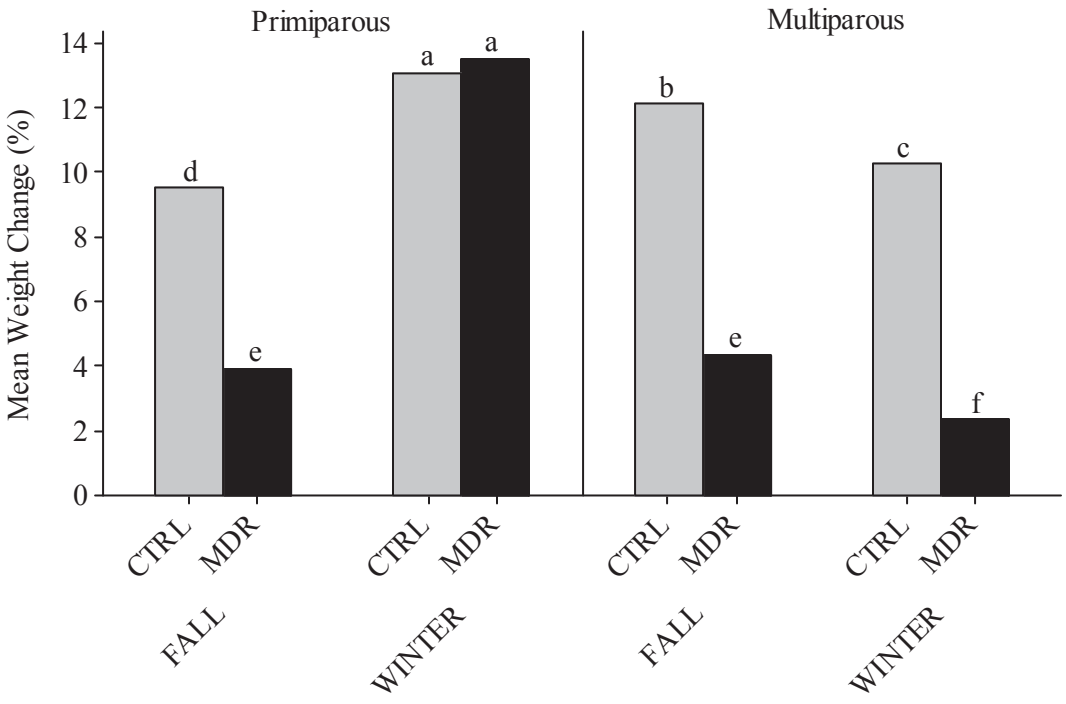

Figure 6. Mean weight gain in the fall and weight loss in the winter of CTRL and MDR female mink separated by parity and expressed as percent body weight. Different letters indicate significant differences ($P<0.05$) between groups and time points. Primiparous females n= 157 in each group for both fall and winter. Multiparous females n=43 in each group for both fall and winter.

5.5 Production and Mortality

As can be seen from the first years' production results (Table 2), breeding results did not differ between the groups with regard to the number of females that were single or double mated (P=0.601). There were also an equal number of unsuccessfully and successfully bred females in each group. The number of females remaining at whelping time, as well as the number of females that did or did not whelp, was also not significantly different between the groups. The litter size data did, however, differ between the groups at day 1 and 21 of lactation (P<0.05) with the MDR females having superior litter sizes at these times. The number of females weaning one or more kits was not significantly different between the groups (P=0.610) but the number of kits from continuing females was significantly higher in the MDR females (P=0.003).

In the second year of the trial there were significantly more (P=0.048) primiparous females in the trial than multiparous females due to removal of pairs that did not meet the criteria to continue (Table 3). The second year results were quite similar to the first year. Breeding results did not differ between the groups with regard to the number of females that were single or double mated and the number of successfully and unsuccessfully bred females was also not statistically different. The number of females remaining at whelping time, as well as the number of females whelping or not, was also not significantly different between the groups. The litter size data showed more statistical significance in favor of the MDR females in the second year (P<0.05). The number of females weaning one or more kits was not significantly different between the groups but the number of kits from continuing females was again significantly higher in the MDR females (P<0.05).

With regard to mortality rates in the first year (Table 4), the treatments did not significantly differ (P=0.118) during the fall, breeding, gestation, and lactation periods. Time did, however, have a significant effect on mortality rates (P=0.009) with significantly higher mortality rates during lactation as compared to fall, breeding, and gestation. Four CTRL and nine MDR dam deaths occurred during lactation/post-lactation. No significant time and treatment interaction effect was found.

Similar to first year's results, the treatments did not significantly differ (P=0.663) in mortality rates during the fall, breeding, gestation, and lactation periods during the second year (Table 5). Time did, however, have a marginally significant effect (P=0.076). Again, as expected, the lactation period tended to result in the highest number of mortalities for both treatment groups. Four CTRL and four MDR dam deaths occurred during lactation or post-lactation. No significant time and treatment interaction effect was found. Parity was also found to be insignificant (P=0.997) with regard to dam mortality.

Table 2. Year one production results of 200 sister-pair mink dams from September 2009 to September 2010.

	CTRL	***MDR***	***P-value***
Trial females	100	100	*1.000*
BREEDING			
Pre-breeding eliminations*	2	2	*1.000*
Breeder females	98	98	
Unsuccessfully bred	3	3	*1.000*
Successfully bred	95	95	
Single Mated	9	7	*0.601*
Double Mated	86	88	
WHELPING			
Pre-whelping eliminations*	2	3	*0.650*
Trial females at whelping	93	92	
Females whelped	76	74	*0.823*
Females did not whelp	17	18	
Total kits born	474	527	*0.094*
Total kits live d1	377	451	***0.010***
Total kits live d21	*354*	*409*	***0.046***
Total kits live d42	*336*	*382*	*0.086*
Females weaned 1+ kits	72	66	*0.610*
Continuing Females	43	43	*1.000*
Kits of continuing females	188	251	***0.003***

* indicates females that were eliminated due to death or illness.

Table 3. Year two production results of 200 sister-pair mink dams from September 2010 to September 2011.

	CTRL		**MDR**		***P-value***	
	Primiparous	***Multiparous***	***Primiparous***	***Multiparous***	***Group***	***Parity***
Trial females	57	43	57	43	*1.000*	***0.048***
BREEDING						
Pre-breeding eliminations*	0	4	0	4	*1.000*	
Breeder females	57	39	57	39		
Unsuccessfully bred	0	1	1	1	*0.565*	
Successfully bred	57	38	56	38		
Single Mated	4	7	6	4	*0.837*	
Double Mated	53	31	50	34		
WHELPING						
Pre-whelping eliminations*	0	0	1	0	*0.327*	
Trial females at whelping	57	38	55	38		
Females whelped	45	32	44	30	*0.798*	
Females did not whelp	12	6	11	8		
Total kits born	272	172	309	216	***0.009***	
Total kits live d1	233	149	269	185	***0.013***	
Total kits live d21	222	118	247	154	***0.025***	
Total kits live d42	207	115	230	148	***0.034***	
Females weaned 1+ kits	42	25	38	25	*0.726*	
Continuing Females	27	17	27	17	*1.000*	
Kits of continuing females	135	75	169	91	***0.021***	

* indicates females that were eliminated due to death or illness.

Table 4. Year one survival counts of 200 sister-pair mink dams from September 2009 to September 2010.

	CTRL		***MDR***		***P-value***	
	Live	Dead	Live	Dead	***Group***	***Time***
Fall	100	0	99	1	*0.118*	***0.009***
Breeding	100	0	98	1		
Gestation	99	1	98	0		
Lactation	95	4	89	9		

Table 5. Year two survival counts of 200 sister-pair mink dams from September 2010 to September 2011.

	CTRL				***MDR***						
	Primiparous		***Multiparous***		***Primiparous***		***Multiparous***		***P-value***		
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	***Group***	***Parity***	***Time***
Fall	57	0	43	0	57	0	43	0	*0.663*	*0.997*	***0.076***
Breeding	57	0	43	0	57	0	43	0			
Gestation	57	0	43	0	56	1	43	0			
Lactation	55	2	41	2	55	1	42	1			

5.6 Body Weight Loss during Lactation

When analyzing the percent body weight loss for the two time intervals during lactation, parity was found to be insignificant (P=0.719). From day 1 to 21 both the CTRL and MDR groups lost less than 3% of their body weight and the groups did not significantly differ (P=0.185) in their percent body weight loss during this interval (Figure 7). From day 21 to 42, the percent body weight loss was far greater than during the first three weeks of lactation. The CTRL and MDR females both had a significant increase in percent body weight loss from the first three weeks to the last three weeks of lactation (P<0.001). At this point in the lactation period the MDR females lost significantly more (P=0.004) body weight (13.32%±0.83) than the CTRL females (9.98%±0.82). As for total body weight loss, the groups did not significantly differ from each other (P=0.201). The total percent body weight loss for the CTRL and MDR females were 12.33%±1.05 and 14.22%±1.06 respectively. The greatest amount of weight loss occurred during the last three weeks of lactation.

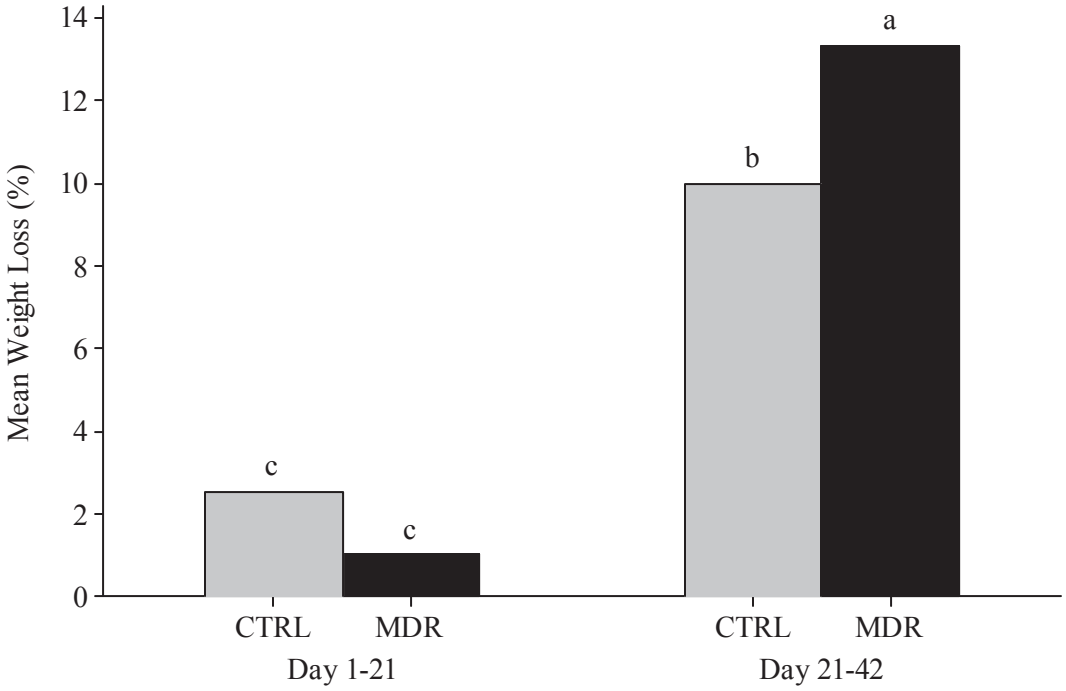

Figure 7. Mean percent body weight loss of CTRL and MDR female mink during lactation. Different letters indicate significant differences ($P<0.05$) between groups and time points. CTRL n= 153 at both time points and MDR n=148 at both time points.

5.7 Reproductive Performance

Total kits born (Figure 8), including live and dead, was found to be significantly higher ($P=0.001$) in the MDR females (7.34 ± 0.60) compared to the CTRL females (4.97 ± 0.41). Initial litter size was not found to be significantly affected by the number of matings or the parity of the female.

With regards to live litter size, the group and mating interaction was found to be significant ($P=0.005$). The results (Figure 9) show that for the MDR group, live litter size is independent of the number of matings ($P=0.574$) with a single mating resulting in 6.32 ± 0.55 kits and a double mating resulting in 6.00 ± 0.15 kits. For the CTRL group, the number of matings did significantly affect live litter size ($P<0.001$) with a double mating resulting in a significant increase, from 3.34 ± 0.37 to 4.96 ± 0.16, in the number of live kits per dam during lactation. The time point during lactation (D1, D21, or D42 pp) and the parity of the female were found to have no significant effect on live litter size.

When comparing the two groups, regardless of whether the females were single or double mated, the MDR group had a significantly higher mean number of kits than the CTRL group ($P<0.05$). The number of mating attempts had no significant effect on the total number of kits raised per female.

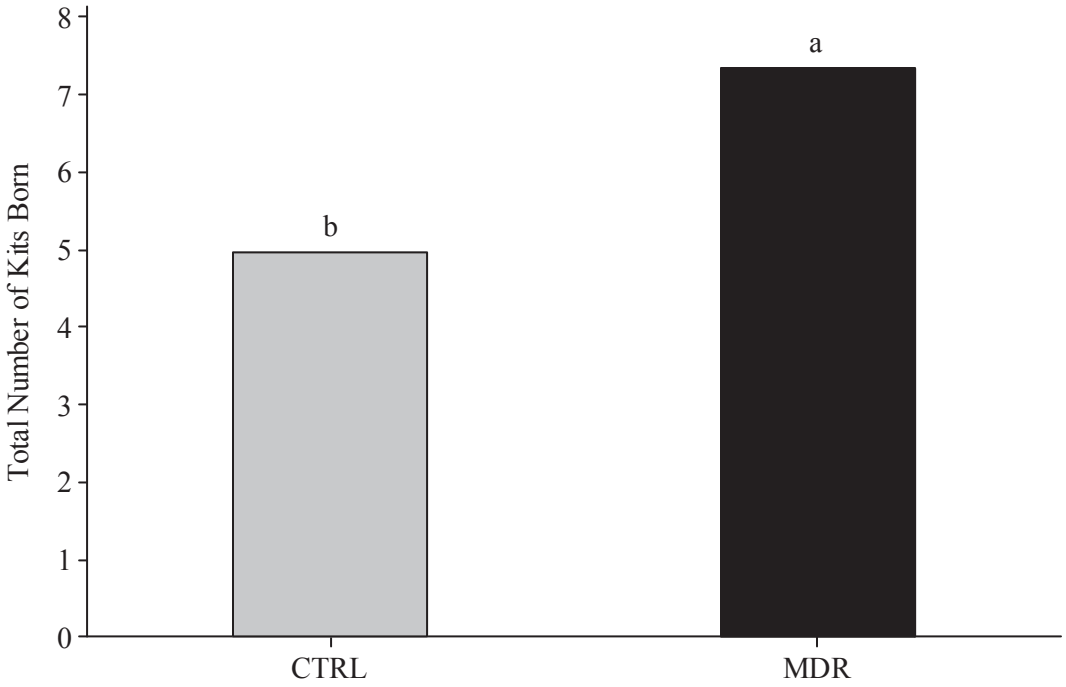

Figure 8. Total number of kits born of CTRL and MDR female mink. a-b indicates significant differences ($P<0.05$) between groups. CTRL n= 153 and MDR n=147.

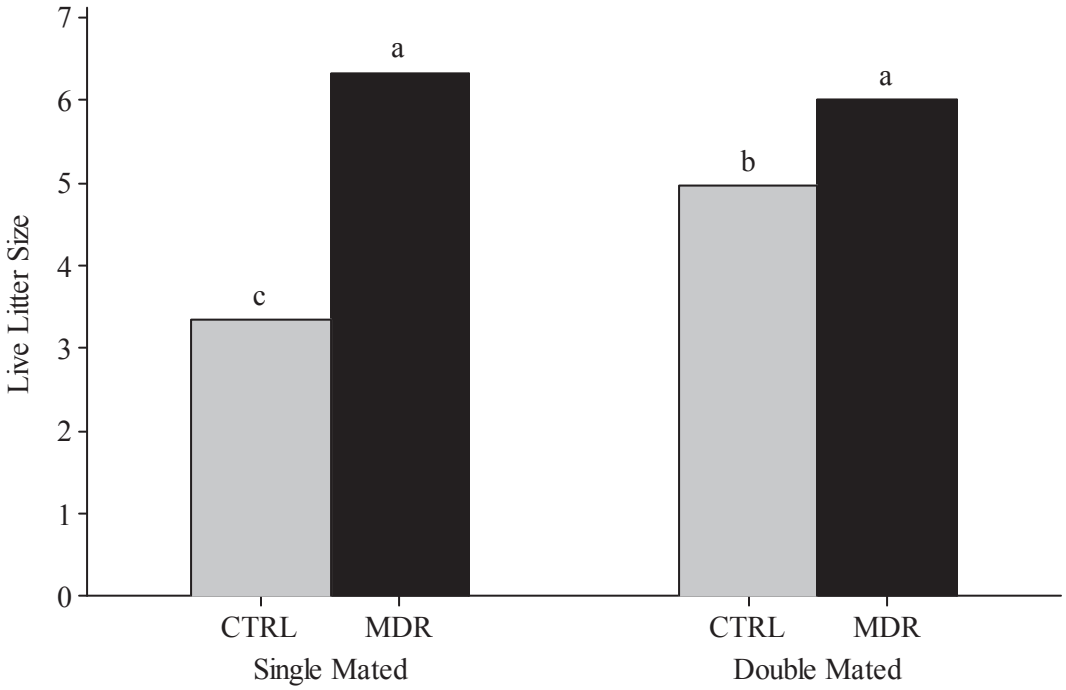

Figure 9. Live litter size of CTRL and MDR female mink. Different letters indicate significant differences ($P<0.05$) between groups and mating occurrences. Single mated CTRL n=45 and MDR n=24. Double mated CTRL n=414 and MDR n=417.

5.8 Comet Assay

The comet assay was successful in determining varying degrees of DNA damage (Figure 10). Regardless of group, the primiparous females increased their comet scores significantly ($P<0.001$) from September when they were first sampled for the comet assay (57.14±4.46), to weaning (108.32±5.38) (Figure 11). Regardless of time point, the mean comet scores were different between the groups with a higher mean comet score in the CTRL group (91.69±4.92) in comparison to the MDR group (73.77±4.92, $P=0.011$) (Figure 12).

As can be seen in Figure 13, the multiparous groups show differences in comet scores at the first weaning with the CTRL females having significantly higher scores (100.76±9.62) than the MDR females (61.09±9.62), indicating higher oxidative damage in the CTRL dams. This difference did not, however, occur at the second weaning. At this point the CTRL females had a mean comet score of 121.91±12.27 and the MDR females had a mean comet score of 140.12±12.27. From the first to the second weaning, the CTRL females did not have increases in comet scores ($P=0.250$) yet the MDR females did ($P<0.001$).

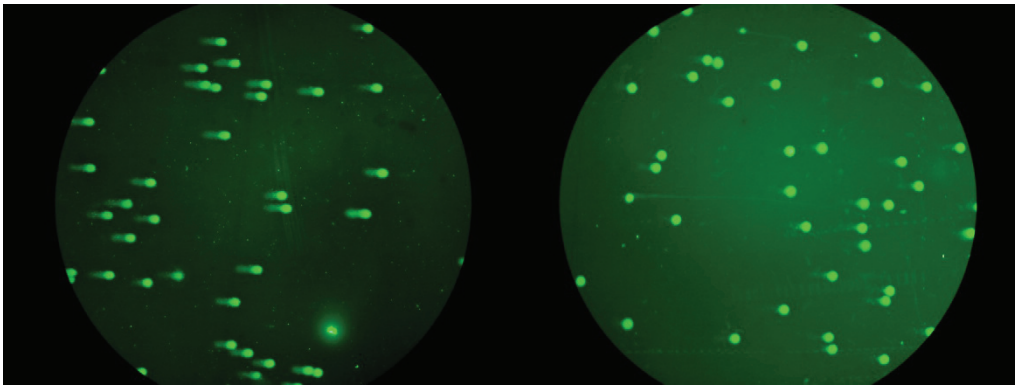

Figure 10. Examples of comet assay images illustrating highly damaged cells on the left from a 2009 CTRL dam at her first weaning, and minimally damaged cells on the right from a 2009 MDR dam at her first weaning.

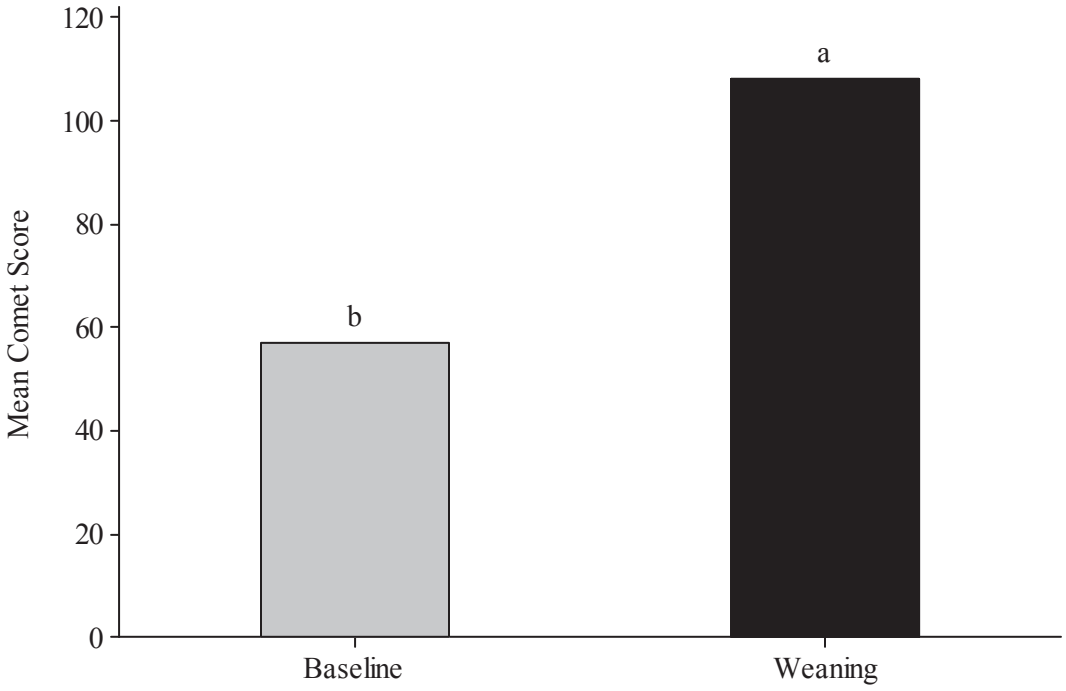

Figure 11. Mean comet scores of primiparous females at the baseline (September) and at weaning (day 42 pp). a-b indicates significant difference ($P<0.05$) between time points. Baseline n=96 and weaning n=72.

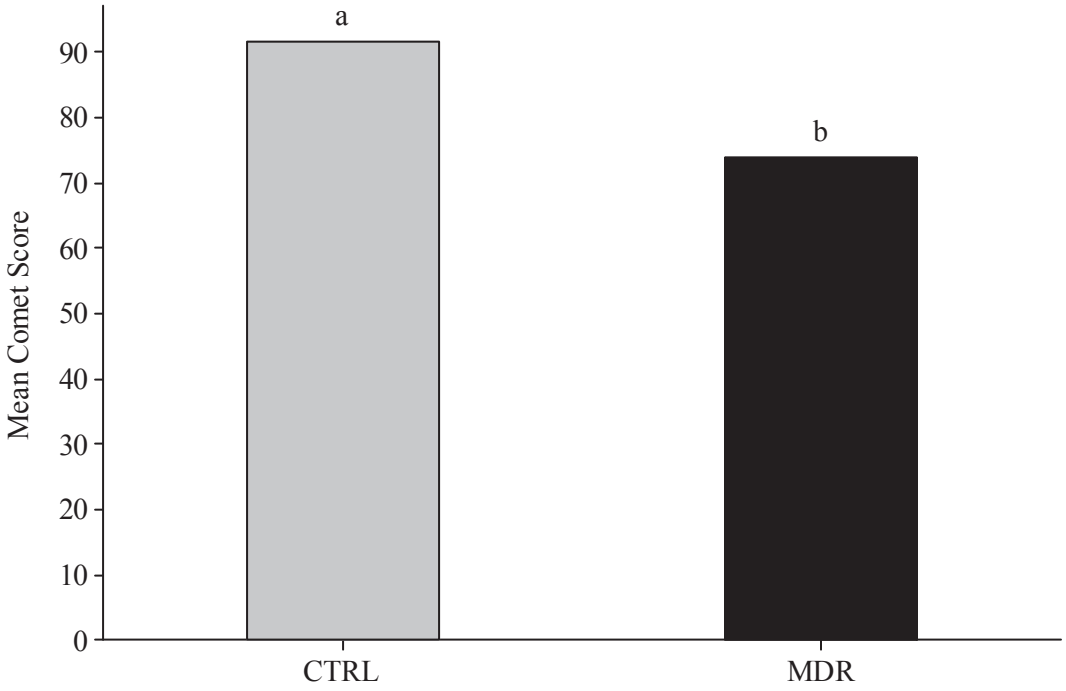

Figure 12. Mean comet scores of primiparous CTRL and MDR females. a-b indicates significant difference ($P<0.05$) between groups. CTRL n=84 and MDR n=84.

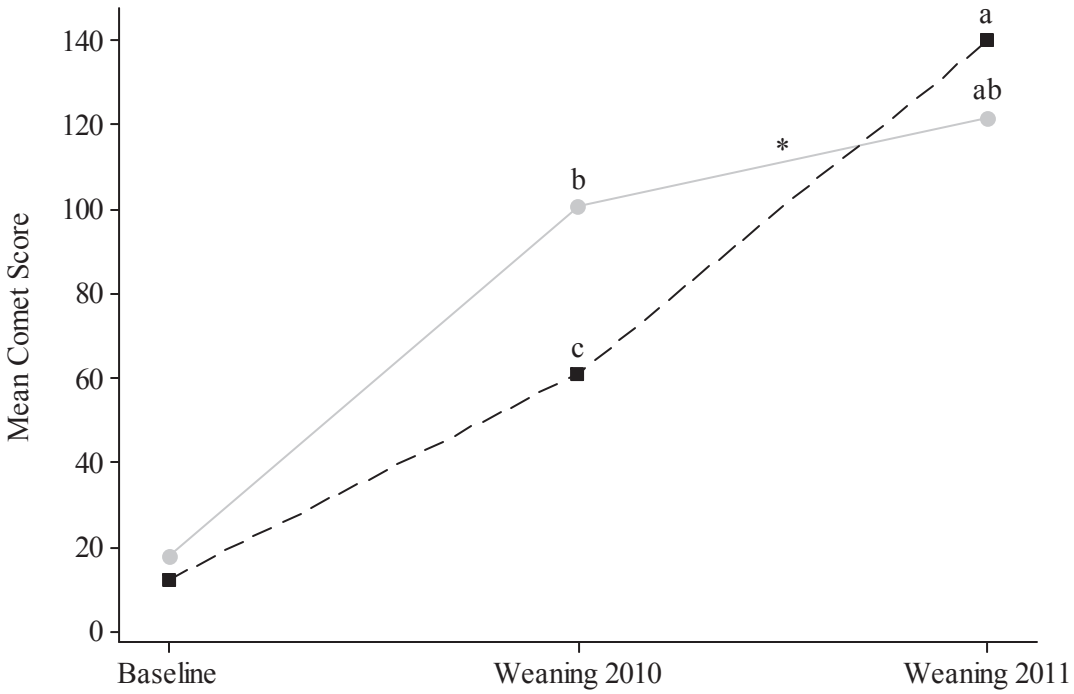

Figure 13. Mean comet scores of multiparous CTRL (•) and MDR (▪) females from September 2009 (Baseline) to June 2011 (Weaning) though two reproductive cycles. Different letters indicate significant differences ($P<0.05$) between groups and time points. * indicates no significant group change in comet score from one time point to another. CTRL n=24 and MDR n=24. Due to statistical analysis restrictions, differences between the groups at the baseline measurement and changes from baseline to the first weaning could not be determined.

5.9 Telomere Assay

There were thirty-nine females born in 2009 who had data available for the three time points: baseline, 1st weaning, and 2nd weaning. These females were included in the repeated measures analysis in which time (P=0.636) and group (P=0.456) were found to be non-significant, indicating no changes in relative telomere length. Next, data from all females born in 2009, whether they had data for one or both weaning periods, was included in the analysis without the repeated measures option (Figure 14). Time was determined to be significant (P<0.001) with the mean baseline relative telomere length significantly increasing (P<0.001) from baseline (1.07) to the first weaning period (2.66). Relative telomere length did not significantly change (P=0.386) from the first weaning period (2.66) to the second (2.86). A trend for significance for the time and group interaction (P=0.058) was also found with respective mean relative telomere lengths of the CTRL and MDR females being 1.02 and 1.05 at the baseline measurement, 1.56 and 1.69 at the first weaning period, and 1.81 and 1.57 at the second weaning period.

The primiparous data analysis also determined time to be significant (P<0.001) and showed an increase in telomere length from when the mink females began on the trial in September to their first weaning (Figure 15).

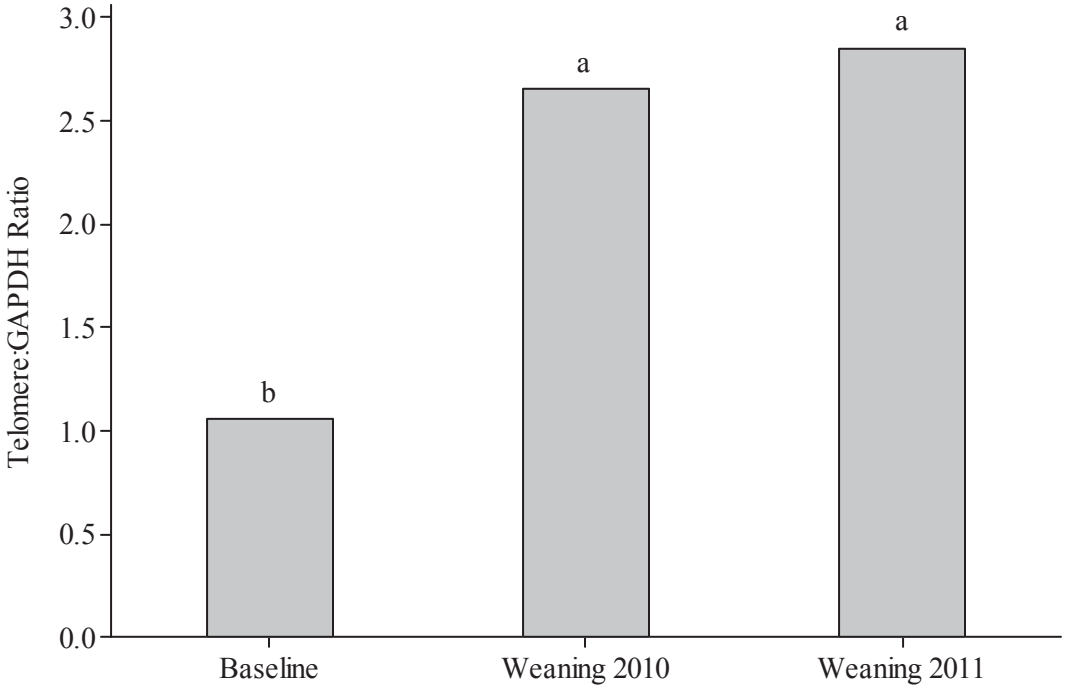

Figure 14. Relative telomere lengths of multiparous females at the baseline (September), and at weaning 2010, and weaning 2011. a-b indicates significant differences ($P<0.05$) between time points. Baseline n=102, weaning 2010 n= 102, and weaning 2011 n=40.

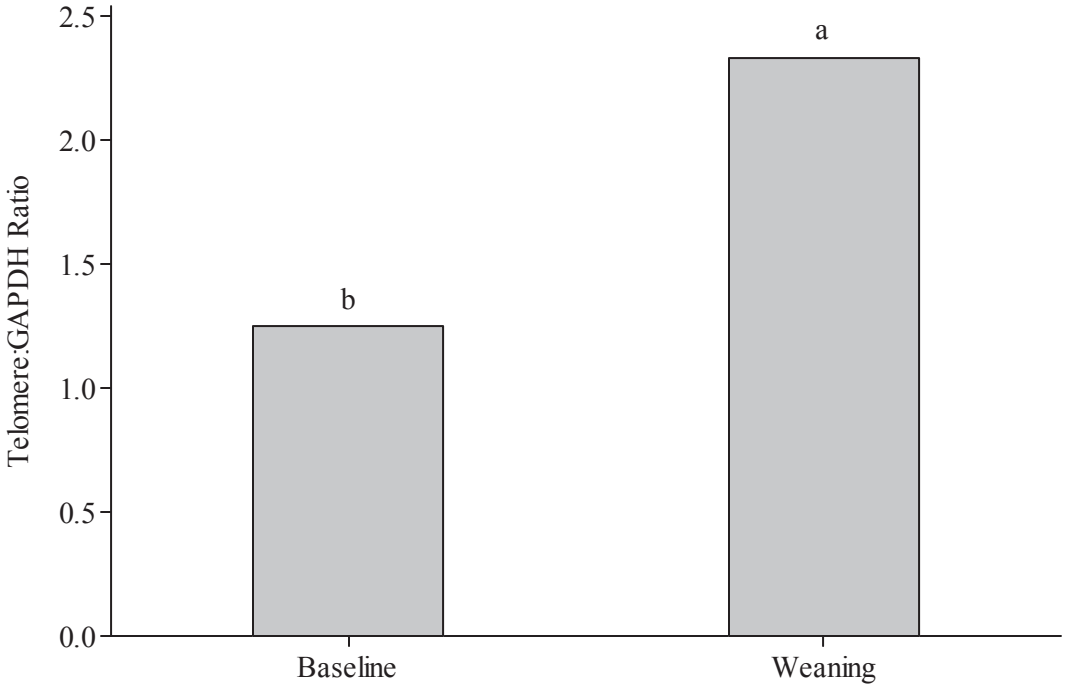

Figure 15. Relative telomere lengths of primiparous females at the baseline (September) and at weaning. a-b indicates significant differences ($P<0.05$) between time points. n=162 for each time point.

CHAPTER 6: DISCUSSION

6.1. Seasonal Changes in Body Weight and Body Condition

Our results showed that MDR of mink breeder females during the fall leads to significantly lower body weights and more ideal BCS than the CTRL dietary regime. Diet restriction also leads to reduced weight gains and losses in preparation for breeding and a better ability to maintain body weight and condition during and post-lactation. This dietary management practice allowed for the maintenance and prevention of drastic fluctuations of weight and BCS in the MDR females. Moreover, the results of this study are in agreement with previous results by Hynes *et al*. (2004) who found a high correlation between body condition score and body weight of mink breeder females. The regression analysis in this study, however, showed a much weaker relationship with R^2 values of 43% whereas Hynes *et al*. (2004) determined R^2 values of 0.98 and 0.99 depending on the month of data collection. Body condition scoring is a subjective measure and so judgment may change from month to month though the scorer's experience should limit this. Properly restraining the mink for precise scoring can also be a challenge. If the mink is not fully extended it may appear and feel to have a larger body condition than what it truly is. Additional factors that may influence BCS may be age and body length. These factors may all in turn affect fat distribution which can ultimately affect the mink's body condition score.

6.1.1 Fall Weight Gain

The significantly larger weight gains seen in the CTRL females during the fall were expected since the MDR females underwent feed restriction at this point whereas autumnal fattening was permitted for the CTRL females. In the wild, mink have

increased feed intake during the fall in order to accumulate subcutaneous body fat which serves as insulation and as an energy reserve during the cold winter months when feed intake by the mink is reduced (Korhonen and Niemelä, 1998; Tauson, 2001). By limiting the feed allowance in the MDR group from September onwards, less excess energy was available for storage as body fat. Body fat accumulation is most likely to occur from September onward as mink will complete most of their longitudinal and physiological development by September (Lagervkvist *et al*., 1993; Rouvinen-Watt *et al*., 2005). Any body weight gains occurring after September are, therefore, likely to be due to body fat deposition.

Maximum body weights occurred in December for the multiparous females and in October for the primiparous females. These results are in agreement with previous studies which determined that the greatest body weights of female mink occur in October or December, followed by body weight loss due to reduced energy intake associated with colder temperatures (Korhonen and Niemelä, 1997; Korhonen, 1990).

6.1.2 Conditioning for Breeding

Excessively high body weights are not conducive to optimal breeding success and the need to restrict feed to slim over-conditioned females prior to breeding is common and known to increase reproductive performance (Damgaard *et al*., 2004; Tauson and Aldén, 1985). The process of slimming results in sudden weight loss and resembles a severe NEB which is akin to fasting. The CTRL females may, therefore, have an increased risk of developing fatty liver as they require more drastic slimming prior to breeding (Bjornvad *et al*., 2004; Rouvinen-Watt *et al*., 2010). The fact that the multiparous MDR females do not have to lose significant amounts of weight prior to

breeding is therefore very beneficial health-wise as these females did not require drastic weight loss prior to mating as they were already at a suitable weight for optimal breeding success.

The percent weight losses determined in this study for all CTRL females and the primiparous MDR females agree with typical pre-mating weight losses observed in captivity which can range from 9-25% (Korhonen *et al*., 2002). The multiparous MDR females, however, lost an average of only 2.33% which is far below the normal range. The minor winter weight loss seen in the multiparous MDR females is desired as it has been found that large weight reductions during the winter result in poor reproductive performance (Tauson, 1993). Plane of nutrition has been linked to reproductive performance with minimal weight reductions in the winter leading to superior reproductive performance compared to females on a high feeding intensity and losing much greater body weight due to the necessary period of slimming prior to breeding (Tauson and Aldén, 1984).

With regard to parity, the primiparous females showed more drastic weight losses during the fall as compared to the multiparous females, and significant differences were observed in BCS of the different parity groups of females during the winter. Slimming may have inadvertently occurred earlier in the fall in the primiparous females due the fact that they were pair-housed with a male until October. This is a conventional housing practice found to optimize growth and fur development but does cause competition for feed as males consume more than females due to their much larger body size (Korhonen and Niemelä, 1997). This housing practice may have also resulted in less accumulation of subcutaneous fat for energy reserves and insulation by the primiparous females as

competition for feed would have existed. Body conditioning at this time may have, therefore, been less successful, or perhaps the feed restriction procedure may have taken time to optimize to attain an ideal body condition and so restriction may have at times been too severe. Both parity groups were at similar BCS until winter when mobilization of energy reserves would have occurred as cold temperatures lead to increased energy demands and feed intake is also at its lowest during this time (Korhonen, 1990). The primiparous females may have had less initial insulation and energy reserves available and so less ability to maintain body temperature and therefore body condition. They, therefore, lost body condition more quickly in the winter than the multiparous females. As for group differences and seemingly drastic weight losses exhibited by the primiparous MDR females, these may also be explained by the smaller amounts of feed fed to the restricted females. These smaller feed portions may have frozen more rapidly than the larger portions of feed given to the CTRL females (Korhonen and Niemelä, 1998). This would further limit intake resulting in an additional and unintentional restriction of feed in the MDR females.

For the primiparous females, differences in body weight were seen between the groups throughout the production year except at day 21 and in August, but these differences were not seen from February onward in the multiparous females. Again, a more severe restriction during the fall may have been the cause which resulted in a maintaining of significantly lower body weights for the remainder of the year as mink are highly responsive to changes in energy supply (Tauson and Forsberg, 2002). Also, the young females may have been more sensitive to changes in feed intake due to their higher metabolic rates, as reported previously for young rats as compared to older rats

(McCarter and Palmer, 1992).

The large amounts of weight gain and loss seen in the CTRL group is a common occurrence seen on mink ranches. This is of great concern as the rapid influx of body fat, which often occurs during the fall in ranched mink, or mobilization, which is a common occurrence during the slimming prior to breeding, may be a key contributor to fatty degeneration of the liver in mink (Hunter and Barker, 1996). After only 5-7 days without food, the American mink enters a state of proteolysis and will develop a fatty liver (Mustonen *et al*., 2005; Bjornvad *et al*., 2004). The period of slimming may therefore resemble a period of fasting. A MDR was successful in reducing these harmful weight fluctuations during the fall and winter. In contrast, the practice of slimming prior to breeding was necessary for the overconditioned CTRL females. The multiparous MDR females showed much less percent body weight loss during the winter which would reduce these females' risk of developing fatty liver. The primiparous MDR females may have lost more weight than the older MDR females as they may not have had as large deposits of subcutaneous fat reserves as most of their feed intake and energy would have been used for longitudinal growth which usually continues until September (Lagervkvist *et al*., 1993). With increasingly cold temperatures, energy intake is naturally lowered and the animals with less energy reserves to sustain homeostasis will be more sensitive to these changes in feed intake (Korhonen, 1990).

Restricting feed during the fall was successfully in generating mink females with body weights and body conditions more conducive to better health during the fall and winter. Feed restriction during this time also reduced the need to incorporate slimming prior to breeding, which can lead to certain metabolic diseases such as fatty liver, as the

restricted females were prevented from accumulating as much body fat reserves as the CTRL females.

6.1.3 Gestation and Lactation

During gestation, both groups gained significant amounts of weight which was expected as the fetuses are growing and developing and the females' plane of nutrition is increased (Korhonen, 1990; Atkinson, 1996). During the first three weeks of lactation, the MDR females were able to sustain their energy requirements via feed intake despite the strain of nursing to maintain their large litters. This is contrary to the CTRL females, which seem to have rapidly entered a state of NEB with weight loss quickly ensuing. This indicates that feed intake was not sufficient to sustain the energy needs of the dam in early lactation, necessitating the mobilization of energy reserves, and potentially placing the CTRL females at higher risk of developing fatty liver disease (Bjornvad *et al*., 2004; Rouvinen-Watt *et al*., 2010). The inability of the CTRL females to maintain their body weights may also be due to the suggested premature aging seen in obese individuals (Barton, 2010). Obese individuals often exhibit many conditions typically associated with aging such as arterial hypertension, diabetes, and atherosclerosis. Hansen and Berg (1998) found greater weight loss in older dams as compared to yearling dams and so the accelerated aging associated with obesity may have led to the CTRL females exhibiting significant reductions in weight, similar to what occurs in older dams. On the other hand, the MDR females showed no significant changes in body weight and so these dams may be benefiting from the anti-aging effects of caloric restriction (Masoro, 2005). A decrease in metabolic efficiency may also occur with age in overfed animals. In a lifelong study of Fischer 344 rats, McCarter and Palmer

(1992) determined that rats fed 60% of *ad libitum* were able to maintain appropriate fluxes of nutrients and metabolic rates during periods of energy utilization as compared to rats fed *ad libitum*. The restricted rats were also able to maintain cellular homeostasis due to this increased metabolic efficiency.

The overall weight loss and loss of body condition seen in both groups during lactation is in close agreement with findings by Tauson *et al*. (1998) who found that after two weeks of lactation, the female is unable to sustain her energy requirements through feed consumption. Fat reserves are therefore mobilized to meet the demands of milk fat provision. This inevitable weight loss during lactation has been previously reported (Hansen and Berg, 1998). The maximum weight loss during lactation occurred in the last three weeks which is in agreement with findings by Hansen and Berg (1998) who found that of the average 15% of body weight that is lost during lactation, the majority, 10% in their case, occurs during the last two weeks. In another case, Clausen *et al*. (1992) found that weight losses could reach as high as 30% which did not occur in this study. Also, during the two years of this study the older dams did not lose more weight than yearling dams, contrary to what was reported by Hansen and Berg (1998). Regardless, these significant reductions in weight during lactation are always a concern as they cause the female to become vulnerable to fatty liver disease and nursing sickness (Wamberg *et al*., 1992; Hunter and Barker, 1996). Furthermore, when the female reaches an energy deficit and she cannot eat enough to sustain body homeostasis, weight is lost and she will enter a NEB. This status has adverse health consequences such as the development of nursing sickness with symptoms being dehydration, emaciation, lethargy, vomiting, and renal dysfunction (Schneider, 1996).

Discrepancies between the two parity groups may be explained by maternal experience. Whelping and lactation are stressful periods for the dam due to the demands of lactation and these periods may be more stressful for the naïve primiparous females. These dams, having no maternal experience, may undergo more stress which could increase stereotypic behaviours and therefore locomotor activity which could lead to lower body weights seen at day 42 of lactation compared to the multiparous females (Svendsen *et al*., 2007). A study by Brink and Jeppesen (2005) found that dams spent more time performing stereotypies as kits grew older which indicates increasing stress and this could be worse in first-time mothers.

From weaning until July the multiparous MDR females were the only group able to maintain their body weight. This shows their resistance to the usual weight loss that accompanies the weaning period. The significant losses in weight in the other groups may be due to the reduced feed intake which often occurs at weaning, an upsetting and stressful event for the dam as indicated by high plasma cortisol concentrations (Hunter and Barker, 1996; Sørensen *et al*., 2001; Clausen *et al*., 1999).

From July to August, the multiparous CTRL females were unable to regain significant amounts of weight. This suggests that their metabolism was unable to recover from this energy demanding time. Recovery did, however, occur in the primiparous CTRL females and in both parity groups in the MDR females. The multiparous CTRL females' inability to regain weight may again be indicative of less efficient metabolism and energy utilization which occurs with age (McCarter and Palmer, 1992). These females are a year older than the primiparous females and the periods of obesity they experience during the production year may cause further degeneration and exacerbation

of the normal aging process. This may have caused accelerated aging of homeostatic systems making these females metabolically older than the multiparous MDR females. Weight gains and increases in body condition post-weaning may have also occurred due to significant reductions in stress seen in mink one week after weaning, which is accompanied by an increase in feed intake (Sørensen *et al*., 2001). Clausen *et al*. (1999) determined that cortisol levels were increased 2-fold two days after weaning followed by a return to unstressed levels one week after weaning.

A MDR during the fall resulted in an improved ability to handle lactational demands as exhibited by the maintenance of body weight during the first three weeks of nursing. It is believed that the outcome of this study has positive implications for the dam's health and also welfare. In addition, this study suggests that there is potential for mink ranchers to reduce production costs as feed, the most costly input, could be reduced.

6.2 Reproductive Performance

This research demonstrated that MDR of mink females during the fall resulted in superior litter sizes throughout lactation in comparison to non-restricted dams. On average, the litter size, including live and dead kits, of the MDR dams was 2.37 kits larger at birth compared to the CTRL dams. These results are in accordance with those previously reported where large weight losses during the winter show negative effects on mink female reproductive performance while limited feed intake increases litter size and reduces kit mortality (Tauson and Aldén, 1984; Korhonen and Niemelä, 1997). The litter sizes reported in this study for the CTRL dams are in close agreement with typical commercial mink farm averages of 3.5-5.0 kits weaned per female bred, while the MDR females produced larger litters (Hunter and Schneider, 1996). Parity was found to be an

insignificant factor which is not in agreement with results by Hansen (1997) who reported that multiparous dams had significantly fewer dead kits than yearling dams. Ślaska *et al*. (2009) also determined that, regardless of coat color, prolificacy was higher in two year old females compared to yearling females. Based on our results, MDR of primiparous or multiparous females from September to December resulted in a larger number of kits born alive or dead and also a larger live litter size throughout lactation.

The MDR females were able to produce significantly larger live litters than the CTRL females even compared to the CTRL females who were mated twice, and as a result, showed increased live litter size. It is remarkable that for the MDR group there was no difference in the live litter size between females that were mated twice in comparison to those that were mated only once. The MDR results are in agreement with Malmkvist *et al*. (1997) who found that females mated once or twice showed no difference in litter size, even between two groups of mink selected for confident or timid behavior. Mating attempts was not a significant factor and so ease of mating is not thought to be a plausible explanation. This result reflects positively on ranch management practice as it is likely to reduce breeding labor as mating is a labor and time intensive duty and may be unnecessary in dietary restricted females. Our findings for the CTRL females are in agreement with Ślaska *et al*. (2009) who reported improved litter sizes in response to multiple matings, especially in yearling dams, for certain color types of mink.

A positive relationship between litter size and weight loss of the dam during lactation has been previously reported yet this is in contrast to our findings where the MDR females produced larger litter sizes yet seemed to be better able to maintain their body weight and body condition during the first three weeks of lactation (Hansen and

Berg, 1998). A greater weight loss would be expected in this case as the MDR females would have increased lactational demands with more offspring to support. An earlier study noted slower weight gains post-lactation in high producing females (Tauson, 1988) which is also not in total agreement with our findings as the MDR females raised larger litters and both parity groups in the MDR females were able to gain weight post-lactation.

The results prove that the dam's nutritional status prior to mating has a marked effect on reproductive performance. As reviewed by Zain and Norman (2008), obesity has been shown to cause anovulation, menstrual irregularities, and reduced conception rates. Miscarriages are also linked to maternal obesity. A state of hyperinsulinemia, common in obese individuals, is thought to play a major role. There are several possible reasons that could explain the reduced litter size in the CTRL females and there are several stages during the seasonal production cycle where these mechanisms could influence litter size. The first stage is from anestrus, when the ovary is inactive and only primordial and primary follicles are present, to proestrus, when the ovaries increase in size and the number of growing follicles is increased (Murphy, 1996). Again, obesity seems to be a state of accelerated aging due to the similarities in metabolic imbalances (Barton, 2010; Tzanetakou *et al*., 2012) and aging has been found to lead to a decrease in the number of follicles (Faddy, 2000). Aging also causes higher rates of atresia of primordial follicles and also slows growth rates of follicles which therefore affects follicle development and ability of the follicle to be recruited during ovulation (Faddy, 2000). Since the CTRL females were over-conditioned during the fall, the hormonal disruptions associated with obesity, discussed below, may also play a role. A MDR may exhibit similar effects to flushing, which necessitates moderate feed restriction for two or

more weeks followed by *ad libitum* feeding. Flushing in litter bearing animals has been found to increase the pre-ovulatory follicle population and so a MDR may do the same (Lefèvre and Murphy, 2008).

The next stage at which differences between the groups may have arisen is during ovulation. Once again, follicle populations as well as follicular development may have been lower in the CTRL females. Incomplete follicle development, which may occur in obese females, may lead to non-ovulatory follicles which degenerate by atresia (Murphy, 1996). Fertilization rates would therefore be lower and this is yet another stage at which differences may arise in favor of the MDR group.

After fertilization, the embryo develops to the blastocyst stage and growth and implantation do not proceed until a favorable uterine environment is reached (Lefèvre and Murphy, 2008). Since embryo survival is inversely related to the length of embryonic diapause, smaller initial litter sizes in the CTRL females may be due to a prolonged delay in implantation due to delayed hormonal maturation of the uterine environment. If conditions are not conducive, embryo implantation may not occur. Stress is also known to cause embryonic loss in mink and the metabolic stress due to obesity that the CTRL females undergo may explain the reduced litter size in this group (Hunter and Schneider, 1996). Dam stress during the pre-implantation stage has been previously found to cause embryo expulsion and obesity may cause a stressful uterine environment (Daniel, 1971). The stress implemented in the study was injection of LH and it was not clear whether it was the stress of repeated injections that caused abortion or whether it was the hormone itself.

Several hormones are known to affect the reproductive axis. With regard to LH,

the release of which is quite sensitive to alterations in metabolic status, severe feed restriction will prevent estrus due to termination of LH release (Tauson *et al*., 2000). Luteinizing hormone release and ovulation were, however, able to be maintained in animals of varying weights and fat content. Perhaps a severe state of over-conditioning behaves similar to a state of under-conditioning and so results in similar hormonal and physiological changes such as lack of ovulation. Once again, a MDR may show similar effects to the process of flushing in that LH release is more synchronized in flushed feeding (Tauson, 1993). Flushing also increased ovulation rate and viability of embryos and these benefits may be amplified in the MDR females.

Obesity is also associated with elevated leptin levels or hyperleptinemia (reviewed by Velloso and Schwartz, 2011). This reproductively important hormone influences embryo development, has direct effects on the hypothalamus and ovary, and may signal attainment of a body condition suitable for reproduction and may therefore promote puberty, estrus, and maturation of the reproductive tract (Chehab *et al*., 1997; Mitchell *et al*., 2005). Nagatani *et al*. (1998) showed that in rats leptin has a regulatory role when it comes to GnRH. Leptin maintains LH pulse frequency and leptin resistance, suggested to occur in obesity (Messinis and Milingos, 1999), may therefore impair its role in releasing LH, critical for ovulation. Excessive levels of leptin can thus disrupt its normal signaling function and impair reproductive performance (reviewed by Brewer and Balen, 2010). Insulin may also play a role as it is important for ovarian steroidogenesis, it promotes follicle growth, and is known to stimulate GnRH and, as a result, stimulates LH release (Tauson, 2001; Pasquali and Gambineri, 2004). A decrease in insulin sensitivity occurs in obese individuals (Dandona *et al*., 2004) which could affect the release of these

hormones necessary for proper reproductive function. The CTRL females may have impaired follicle development due to insulin resistance and so follicles may not have been fully developed prior to the first mating. By the second mating, 8-10 days later, there may have been an increased number of fully developed follicles, capable of ovulation, leading to increased litter size for the double-mated CTRL females.

Insulin also stimulates androgen production, a sex hormone that can influence reproductive processes (Douglas *et al*., 2006). The sex hormone-binding globulin is responsible for transporting androgens and this carrier protein is decreased in obese individuals leading to an increase in free androgen and hyperandrogenism is the consequence (Pasquali, 2006). Hyperandrogenism leads to menstrual disturbance as it disrupts normal follicular development and disturbs ovarian function (Ramsay *et al*., 2006). The over-conditioning of CTRL females during the fall may lead to long-lasting hormonal disruptions associated with hyperleptinemia, hyperinsulinemia, and hyperandrogenism. These may negatively affect reproductive performance at all stages of the seasonal cycle from puberty through to whelping.

Larger live litter sizes in the MDR females may also be indicative of other physiological differences between the groups including the ability to better adapt to the metabolic transition to lactation resulting in reduced kit mortality. Hernandez *et al*. (2012) found that female Wistar rats fed on a high-fat diet displayed delayed lactation as compared to females fed a low-fat diet, and mammary glands from these females showed few and small alveoli. Mammary gland messenger ribonucleic acid (mRNA) expression of important milk protein genes was also decreased in the high-fat diet females during lactation. This is in agreement with results found by Flint *et al*. (2005) who also noted

that during lactation, obese mice mobilized greater amounts of adipose tissue and showed more abnormalities in mammary gland alveolar development than lean control mice. They also noted an accumulation of lipids in the secretory epithelial cells which may explain the impaired milk production. Another study on rats found that over-conditioned females had reduced milk production and reduced litter growth as compared to control females. There were also more difficulties during parturition and pups from obese rats showed increased mortality rates (Rasmussen, 1998). Impairments in the physiological mechanisms which control metabolic fuel distribution are implicated as noted in a study by Shaw *et al*. (1997) who failed to see the expected reduced plasma insulin concentrations in rat dams fed a high-fat diet. In C57/B6 mice, milk triglycerides were reduced in obese females compared to the lean controls (Wahlig *et al*., 2012). Dewey *et al*. (2003) also indicated obesity as a risk factor for delayed onset of lactation in humans and this delay can lead to excessive weight loss in the infant, dehydration, and even death. It is possible that the CTRL dams, which were maintained in a heavier body condition, may have had poorer milk let down and mothering ability resulting in smaller litter sizes. The causes of early kit mortality thus warrant further investigation.

In agreement with our findings, Tauson and Aldén (1984) have previously shown that a larger loss in weight during the winter has detrimental effects on reproductive performance of the mink female. They also encourage early selection of breeding stock (August) and implementation of limited feed intake to increase reproductive performance and reduce kit mortality. Korhonen and Niemelä (1997) also found that restricted rations in female mink lead to improved whelping results compared to females on a standard ration. Also in support of our findings are the reports of infertility in obese women

followed by improvements in this respect due to weight loss (Zain and Norman, 2008; Brewer and Balen, 2010).

It was hoped that this more ideal condition in the MDR females would prevent the ill effects of maternal over-nutrition such as those seen in humans. These include, for example, congenital malformations and dystocia (Yogev and Catalano, 2009). However, it is also important to note that maternal obesity can negatively affect the offspring during the pre- and post-natal period with regard to health and survival and also doubles a child's chances of developing adult obesity (Whitaker *et al*., 1997; Villamor and Cnattingius, 2006; Yogev and Catalano, 2009). Further supporting our results is a study conducted by Lagerkvist (1997) who found that litter size did not affect fur quality, one of the most important factors influencing the sale price of the mink pelt. Increasing litter size also resulted in the greatest economic gain (Lagerkvist, 1997).

As expected, mortality of the mink dams was the highest during the stressful and energy demanding lactation period for both treatment groups. During this time, the dam is nursing her large litter while trying to maintain her own body reserves. Unfortunately an equilibrium cannot always be reached between nursing and the dam's own energy demands so she can enter a severe NEB with death commonly occurring soon after. This energy deficient state is thought to be the cause of nursing sickness, a leading cause of death in female mink (Rouvinen-Watt, 2003). The highest mortality rates have been previously reported during the lactation period and the greatest cause of mortality attributed to nursing sickness (56%) followed by mastitis, metritis, and dystocia (Schneider and Hunter, 1993). Although specific diagnoses were not obtained upon post-mortem examination of these females, it is presumed that the dams suffered from nursing

sickness, since this disease is a frequent cause of mortality during late lactation.

It is likely that MDR of mink breeder females during the fall will have a significant economic advantage on the profitability of a mink farming operation by increasing the number of marketable offspring, without negatively impacting pelt quality. Significantly reduced labor would also ensue as the MDR females did not require a double mating to produce larger litter sizes.

6.3 Oxidative Stress and Aging

As the females of both groups aged, their comet scores increased. As organisms age they undergo a biological decline and the deteriorative changes that occur accumulate and reduce the cells' abilities to withstand stress making them more susceptible to damage, disease, or loss of function. The ability to maintain homeostasis is lost and the viability of an organism is compromised (Masoro, 2005; Yu, 1996). Many studies suggest that, due to their harmful effect, ROS, generated during normal metabolism, are a key component in the process of aging (Beckman and Ames, 1998). When free radical production exceeds the cell's antioxidant abilities, oxidative stress occurs and this damage accumulates over time. In agreement with these results, Higami *et al*. (1994) used the comet assay to analyze male rat hepatocytes and found that the migration length of DNA and proportion of highly damaged cells was significantly increased in older rats as compared to young rats. They suggest that as the rats age, so does their vulnerability to oxidants. Their redox status therefore becomes altered. This may be due to a weaker anti-oxidative defense system or an increase in the overall production of ROS (reviewed by Chung *et al*., 2009). Over-conditioning also results in increased oxidative stress which has consequences to reproduction as ROS play a role with regard to the female

reproductive tract. ROS can affect oocyte maturation and follicle development, fertilization and implantation, the development of the fetus, and pregnancy itself by causing abortions, birthing complications and defects in the offspring (reviewed by Agarwal *et al*., 2005). Oxidative stress, which was often significantly higher in the CTRL females as indicated by higher oxidative damage, may play a role in infertility and may explain the smaller litter sizes seen in the CTRL females. Lactation and weaning contribute additional stress to the mink dams (Sørensen *et al*., 2001) and perceived stress, as shown in humans, is significantly associated with higher oxidative stress (Epel *et al*., 2004). Increases in oxidative stress during the lactation period may be more to blame for the increases in comet scores with time rather than age. In a study of mice subjected to a prolonged lactation period, apoptosis increased as lactation proceeded and levels of oxidative damage to mtDNA were continuously elevated during lactation (Hadsell *et al*., 2006). Increases in ROS have also been shown in ewes as a result of progressing lactation (Piccione *et al*., 2008).

Recent rodent studies on the effects of caloric restriction have produced consistent and repeatable results, showing that a decrease in oxidative damage due to a decrease in free radical production by the mitochondria seems to be the primary reason for increased longevity (Gredilla *et al*., 2001; reviewed by Gredilla and Barja, 2005). A similary study, also found reduced oxidative damage in calorie restricted rats (Chung *et al*., 1992). With regards to over-nutrition, often occurring in ranched mink, an earlier study by Collins *et al*. (1998) incorporated the comet assay to assess DNA damage and found that BMI in diabetic patients was positively correlated with DNA strand breaks. A more recent study by Demirbag *et al*. (2005) also used the comet assay to assess DNA damage and

confirmed that human obesity results in higher DNA damage scores than non-obese individuals. Trevisan *et al*. (2001) also determined increasing body mass to be associated with increased oxidative stress and reduced antioxidant potential. The results of these studies agree with the current findings that the calorie restricted females show a trend towards lower comet scores than the CTRL females and did show significantly lower scores at the first weaning period.

It is also likely that the CTRL females exhibited an obese phenotype during the fall and obesity is said to accelerate aging (Tzanetakou *et al*., 2012). Furukawa *et al*. (2004) also found an increase in ROS production in the adipose tissue of mice and Mohanty *et al*. (2002) found that fat and protein intake increase ROS generation by polymorphonuclear leukocytes. Both the process of aging and obesity in the CTRL dams would therefore explain the increased DNA damage in these dams. It is likely they experienced more severe oxidative stress. The MDR dams may also show lower DNA damage due to increases in antioxidant enzymes in these dams or decreases in these enzymes in the CTRL females. This would be in agreement with findings by Meydani *et al*. (2011) who showed increased activity of the antioxidant enzyme glutathione peroxidase in calorie restricted humans. However, obesity is thought to be a state of accelerated aging and a previous study actually found increases in the antioxidant enzymes SOD and catalase with age (Rizvi and Maurya, 2007). Since these two studies on antioxidant enzymes are conflicting with regards to our findings it is difficult to justify which specific mechanism can be attributed to the differences seen between the groups. Even more difficult to explain is the significant increase in score for the multiparous MDR females from the first to the second weaning. Perhaps the stress inflicted on the

MDR females, due to two seasons of producing larger litter sizes than the CTRL females, no longer possess the ability to sustain their previous DNA integrity. Perhaps antioxidant defense mechanisms have become less efficient or have deteriorated in some way. Age has been known to increase vulnerability to oxidants in rats and as aging progresses cells become more susceptible to damage (Higami *et al*., 1994; Yu, 1996; Masoro, 2005). This is perhaps due to the decline in the DNA damage response and repair capacity that occurs with time (Jackson and Bartek, 2009). Another explanation is that in the alkaline assay, when DNA is denatured, replicating structures appear as strand breaks and so these could be interpreted as damaged cells when they are not (Fairbairn *et al*., 1995).

Overall, results of the comet assay suggest that MDR may enhance the integrity of the DNA resulting in less strand breaks and lower comet scores. This dietary management practice seems to have alleviated the consequences to DNA of a stress-inducing state of obesity.

Along with reduced DNA integrity, a decrease in telomere length in peripheral blood cells over time and as aging progresses was expected to occur in the mink dams, as has been shown in numerous species (reviewed by Nakagawa *et* al., 2004). This was anticipated as the cells sampled were not ones known to possess steady telomerase activity such as germ cells (Zimmermann and Martens, 2008). However, the opposite was found in both the primiparous and multiparous females which showed a significant increase in relative telomere lengths over time, except in the multiparous dams from their first to second weaning. These results are also in contrast with findings by Heidinger *et al*. (2012) who determined that reproduction resulted in accelerated telomere shortening in zebra finches.

In human studies, telomere elongation is not a new finding. Elongation of telomeres in humans has been previously reported (Gardner *et al*., 2005; Martin-Ruiz *et al*., 2005; Aviv *et al*., 2009). Nordfjäll *et al*. (2009) sampled 959 human subjects at ten year intervals and used real-time PCR to determine telomere length. They found a reduction in telomere length over time in the majority of people with the fastest telomere attrition rates found in those with longest telomeres at the baseline measurement. Large differences between individuals were also observed. A third of these subjects, however, displayed telomere stability or elongation and these subjects were those with the shortest telomeres. They attribute the differences in telomere attrition rate over time to a preference for shorter telomeres by the telomere maintenance machinery in order to protect the shortest telomeres. Telomere elongation, followed by reduction, was also found in the granulocytes and naïve T cells of divers exposed to oxidative stress in the form of hyperbaric oxygen along with intense physical activity (Shlush *et al*., 2011). The control group was also exposed to the extreme exercise, but not the hyperbaric oxygen, and also displayed a lengthening of telomeres. It is suggested that these results are due to telomerase activation in undifferentiated T cells. Akbar and Vukmanovic-Stejic (2007) determined that the capacity of telomerase activation is highest in undifferentiated T cells and lowest in differentiated T cells. As for granulocytes, since these are terminally differentiated cells, repopulation of peripheral hematopoietic cells with undifferentiated cells, which possess longer telomeres than older and differentiated cells, is thought to be the cause of perceived telomere elongation rather than telomerase activation as granulocytes possess very low or no telomerase activity (Weng, 2001).

During oxidative stress, depletion of the existing granulocyte pool may occur and this may be compensated by an influx of granulocytes with longer telomeres to repopulate the pool (Shlush *et al*., 2011). Lactation is a maternally metabolically stressful period and has been shown to increase oxidative damage to mtDNA in the mouse mammary gland with prolonged lactation leading to apoptosis (Hadsell *et al*., 2006). Adrenocorticotropic hormone and corticosterone, both hormones released via the hypothalamic pituitary adrenal axis in response to stress, are quickly increased during suckling in the rat (Walker *et al*., 1992). At the conclusion of lactation in ewes, an increase in oxidative processes is observed. It is suggested here that the mobilization of lipids and the increased production of glucose, which occur due to a maternal NEB, increases the production of free radicals and so elevating oxidative stress (Rouvinen-Watt, 2003). Piccione *et al*. (2006) also showed significant increases in ROS as lactation progressed in dairy ewes. They conclude that there is an increase in ROS during exhaustive processes such as lactation and so the state of oxidative stress is related to production level. They also observed an increase in oxidative processes as lactation advanced and they propose this is due to an increase in thyroid hormones. Thyroid hormones increase oxygen consumption and accelerate basal and oxidative metabolism by inducing certain mitochondrial enzymes. It is also known that abnormally high levels of thyroid hormone hasten the production of free radicals and cause changes in antioxidant defense mechanisms (Alicigüzel *et al*., 2001; Asayama and Kato, 1990).

The mechanism known as alternative lengthening of telomeres is another prospect. It has been determined that, despite absence of telomerase activity, immortalized cell lines and tumors possess heterogeneous and long telomeres. How these

mechanisms work is not well understood yet recombination is thought to play a role (Bryan and Reddel, 1997; Henson *et al*., 2002). Single-stranded DNA of one telomere end may invade another telomeric terminus and use it as a template for copying which would result in an increase in telomere length. Though this mechanism has been repeatedly found in immortalized human cell lines such as fibroblasts, mice lacking telomerase activity due to a knockout mutation were able to elongate the germinal center lymphocyte telomeres (Herrera *et al*., 2000; Henson *et al*., 2002). This study shows that telomerase activity is not the only mechanism by which telomeres can become longer and so this mechanism should not be disregarded in this case of telomere elongation.

The normal fluctuations in telomere length of a cell population may also be the source of elongation. Svenson *et al*. (2011) suggested a pattern of telomere length oscillation in human blood cells where telomere shortening will occur over a life time yet not in a linear manner. Blood cells are also reflective of immune cell subpopulations and the immune system status, including cell proliferation rate and cytokine level, fluctuates within an individual. Certain cytokines, such as IL-6 and insulin-like growth factor (IGF), have been implicated in the stimulation of telomerase activity and so the immune system status can ultimately affect telomere length (Akiyama *et al*., 2002). This would be of particular concern in the CTRL females as their harmful metabolic states, such as obesity, have been found to increase certain inflammatory mediators such as IL-6 and TNF-α. These findings may explain the CTRL females' significant telomere elongation from the first to the second weaning (Shoelson *et al*., 2007). Another possible explanation are the increases in maternal circulating IGF, an important polypeptide when it comes to nutrient availability and growth of the conceptus, during the first half of pregnancy in humans,

rabbits, and guinea pigs (Sferruzzi-Perri *et al*., 2011). In women these levels continue to increase as pregnancy progresses. In addition, many species exhibit increases in maternal concentrations of IGF- 2 with advancing gestation with the placenta being one of several sources of production. These increases in IGF during gestation may therefore cause up-regulation of telomerase, as mentioned previously, resulting in telomere elongation. All elongation findings in this study may also be explained by an increase in the production of the cytokine IL-6 due to physical or psychological stress as is seen in rats (Zhou *et al*., 1993). The mink dam is likely to undergo physical stress due to her probable NEB, and psychological stress is also likely once she is separated from her litter. Another possibility for telomere elongation in granulocytes is upregulation of telomerase in the progenitors of granulocytes subsequent to a period of oxidative stress, though this has not been studied to date (Shlush *et al*., 2011).

This result of telomere elongation may also be a misleading finding. Baerlocher and Lansdorp (2003) determined telomere lengths in subsets of human nucleated blood cells and found that different lymphocytes possessed different telomere lengths and so telomere lengths in these cells are not homogeneous. Telomere length is therefore reflective of the types of cells present in the sample. Blood cells were chosen for telomere length determination in this case for their ease of obtainment. Studies were previously successful in showing that factors such as life style and life stress do in fact influence leukocyte telomere length with these changes being able to be detected (Epel *et al*., 2004; Cherkas *et al*., 2008). Many telomere length studies utilize these cells for telomere length determination (Frenck *et al*., 1998; Cherkas *et al*., 2008).

It is interesting that for the multiparous females, from the 1st to 2nd weaning, there is no significant change in telomere length. A decrease may have occurred but due to all of the possible reasons listed above, they may have been elongated to their previous weaning's length. Also, perhaps the change was so small that it was undetectable. It would have been beneficial to take samples more frequently, such as after weaning during the fall or winter to have a better understanding of the telomere maintenance or attrition pattern in mink during the strictly seasonal production cycle.

The two genetic markers of oxidative stress and aging were successful in determining the oxidant status of the mink dams. The comet assay indicates higher oxidative damage in the CTRL females compared to the restricted dams and increasing oxidative damage in both groups as aging progressed. The telomere assay elongation results, however, are more difficult to elucidate though telomerase up-regulation is a justifiable factor. Regardless, MDR of mink breeder females improves DNA integrity implying a better overall health status due to the reduced risk of disease associated with reduced DNA damage.

CHAPTER 7: CONCLUSION

In summary, MDR was successful in maintaining more ideal body weights and BCS during the fall in the MDR females. The primiparous and multiparous CTRL females had to be significantly slimmed prior to breeding and the largest gain in fall and loss in winter was seen in the multiparous CTRL females. The MDR females were able to maintain their weight during early lactation while the CTRL females lost significant amounts of weight. During late lactation all females lost weight. From weaning to July, only the multiparous MDR females were able to maintain their body weights. All females regained weight from July to August except for the multiparous CTRL females. The BCS showed a similar pattern during lactation. The MDR females' scores increased from July to August while the CTRL females' scores did not. The groups did not differ in their percent weight loss during the first three weeks of lactation which was followed by a larger loss in the MDR females during the last three weeks. The total loss did not, however, differ between the treatments and the MDR females were able regain body weight and condition post-lactation.

As for litter size, MDR of yearling females during the fall resulted in litter sizes 2.37 kits larger than the CTRL females. Also, regardless of matings, the MDR females showed larger litter sizes while the CTRL females' litter size was influenced by the number of matings. Double mating in the CTRL females still did not lead to as large a litter size as the MDR females.

When comparing the level of oxidative stress among the treatments, the primiparous MDR females had overall lower comet scores and the multiparous MDR females had lower comet scores than the CTRL females at their first weaning. This

indicates that MDR may enhance the integrity of the DNA resulting in less strand breaks. Telomere elongation was seen in all females from baseline measurement to weaning which may reflect possible up-regulation of telomerase.

To conclude, this research has lead to enhanced reproductive performance of mink breeder females and will result in significant economic advancement in the fur industry. It has also enabled us to develop a better understanding of the impacts of body condition on genomic health and reproductive longevity in mink females as very little information exists about these effects in mink. It would be interesting to determine whether a MDR has an effect on the fur quality of mink breeder females as pelt quality is also a major determinant of profitability. A look into the specific physiological and hormonal mechanisms of increased litter size in the MDR dams would be helpful in the interpretation of the litter size results. Also, determination of antioxidant capacity in these dams would clarify whether differences do indeed exist between the groups.

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APPENDIX 1

Feed Analysis Report Values

Table 6. Year one feed analysis report values from September 2009 to August 2010.

Time Period	**September- November**	**December- April/May**	**April/May- June**	**July- August**
Stage of Production	**Late Growth/ Furring**	**Breeding/ Pregnancy**	**Lactation/ Nursing**	**Early Growth**
Dietary Composition				
Moisture (%)	66.32	69.53	68.96	68.27
Solids (%)	33.68	30.47	31.04	31.73
Ash Wet Weight (%)	2.38	2.78	2.55	2.28
Ash Dry Weight (%)	7.12	9.11	8.24	7.18
Protein Wet Weight (%)	13.24	12.99	13.02	12.80
Protein Dry Weight (%)	39.34	42.63	41.99	40.34
Fat Wet Weight (%)	10.08	6.33	7.32	8.79
Fat Dry Weight (%)	29.87	20.80	23.46	27.73

Table 7. Year two feed analysis report values from September 2010 to August 2011.

Time Period	**September- November**	**December- April/May**	**April/May- June**	**July- August**
Stage of Production	**Late Growth/ Furring**	**Breeding/ Pregnancy**	**Lactation/ Nursing**	**Early Growth**
Dietary Composition				
Moisture (%)	66.30	67.56	65.30	63.74
Solids (%)	33.70	32.44	34.70	36.27
Ash Wet Weight (%)	2.61	3.01	3.05	2.66
Ash Dry Weight (%)	7.75	9.29	8.79	7.34
Protein Wet Weight (%)	12.95	12.85	14.35	13.87
Protein Dry Weight (%)	38.50	39.54	41.36	38.24
Fat Wet Weight (%)	9.05	6.90	7.77	9.36
Fat Dry Weight (%)	26.85	21.18	22.37	25.82