THE EFFECTS OF AGE AND PHYSICAL ACTIVITY ON CARDIAC MITOCHONDRIAL TEMPLATE AVAILABILITY

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THE EFFECTS OF AGE AND ~~EXERCISE~~ PHYSICAL ACTIVITY ON CARDIAC MITOCHONDRIAL ~~DNA~~ TEMPLATE AVAILABILITY

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ABSTRACT

Chronic low intensity physical activity may provide a means of countering the age-associated reductions in cardiac oxidative capacity. The purpose of this study was to examine the effects of aging and physical activity on heart mtDNA template availability. Young (n = 6), mature (n = 12) and senescent (n = 12) C57BL6 mice were used in this study with half the mature and senescent animals being allowed voluntary access to exercise wheels. Cardiac DNA was isolated and the mitochondrial DNA content was assessed by slot blots with a mtDNA specific probe containing 574 base pairs of the cytochrome oxidase I gene. Mitochondrial DNA template availability increased in mouse cardiac tissue with senescence. This was reflected in increases in mitochondrial DNA content per milligram of cardiac tissue and mitochondrial DNA relative to nuclear DNA ratios($P \leq 0.05$). Oxidative capacity, as determined by citrate synthase activity, was not significantly changed with age. Compromises to the energy transducing pathway due to aging responses in cardiac tissue may have resulted in an increased rate of mtDNA replication in an effort to produce more mitochondrial specific proteins. Mature mice responded positively to the voluntary chronic physical activity, displaying increased mitochondrial DNA to nuclear DNA ratios ($P \leq 0.05$) and elevated citrate synthase activities ($P \leq 0.05$). Thus the increasing metabolic demand put on the mitochondrial system was associated with elevated mtDNA template levels in these animals. In contrast, the senescent animals demonstrated no relationship between elevated metabolic demands and mtDNA template. This observation in senescent animals may be due to low running velocities and therefore low relative workloads in this age group.

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INTRODUCTION

Numerous investigators have established that with increasing age there is a pronounced decrease in oxidative capacity in mammalian heart and skeletal muscle tissues (Hansford and Castro 1982). These decreases have been attributed to several factors including declines in mitochondrial volume and enzyme activities (Abu-erreish and Sanadi 1978). Ultimately the changes in the mitochondrial protein products must be related to alterations in transcription of mitochondrial genes in the mitochondrial and nuclear genomes. In addition, alterations in mitochondrial DNA (mtDNA) template availability, alterations in mtDNA structure and/or accumulations of alterations (mutations/deletions) in mtDNA templates may contribute to the reduced oxidative capacity accompanying aging. There have been some suggestions that the amount of mitochondrial protein products is directly related to the amount of mtDNA template (Williams *et al* 1987; Williams & Harlan 1987). Williams *et al* (1987) demonstrated that the variation in mtRNA content was directly proportional to that of mtDNA copy number in young animals following nerve stimulation in rabbit tibialis anterior muscles.

The mammalian mitochondrial genome has been characterized as a closed, circular, double stranded DNA structure consisting of approximately 16 kilobase pairs. This is small compared to the nuclear genome from which the bulk of mitochondrial proteins are encoded and subsequently transported into mitochondria through a number of complex transport mechanisms. Mitochondrially encoded subunits are nevertheless essential for the formation of functionally intact respiratory complexes. The assembly of a functioning mitochondrial

energy transducing system depends on the coordinated expression of the nuclear and mitochondrial genetic systems (Hood *et al* 1990).

Gadaleta *et al* (1990) found mitochondrial specific RNA products (subunit I of cytochrome oxidase) to be significantly reduced with aging in heart and that this reduction could be reversed with addition of acetyl-L-carnitine to stimulate energy metabolism. They concluded that the decreased mtRNA was due to a reduced mtDNA transcription rate (Fernandez-Silvia *et al* 1991) which might be caused by a reduced template availability due either to a lower content of mtDNA (in relation to total DNA) or to an alteration of mtDNA structure. In a subsequent study, Gadaleta *et al* (1992) found an increase in mtDNA template in the heart during aging. This study demonstrated that mtDNA copy number is not responsible for the reduced mtDNA transcription in aged heart tissue. However, both increases and decreases have been reported in studies examining the effects of aging on mtDNA template content (Massie *et al* 1975, Stocco *et al* 1978, Gadaleta *et al* 1990 & 1992, Asano *et al* 1992, Takasawa *et al* 1993). Inconsistencies may have resulted from differences in the animal used, tissues examined, or methods used to measure mtDNA template content. In addition, the low percentage of mtDNA deletions/mutations found in aged tissues makes it highly unlikely that they are responsible for the reduced transcription (Gadaleta *et al* 1992, Corral-Debrinski *et al* 1992, Simonetti *et al* 1992, Arnheim and Cortopassi 1992). It has been suggested that the decreased mtDNA transcription may be best explained by metabolic alterations and that an increase in ATP production could provide sufficient energy for the production of more supercoiled mtDNA molecules (Gadaleta *et al* 1990).

The purpose of this study was to examine the effects of aging on the mouse heart mtDNA template in young, mature and senescent mice. Senescence is defined as the process or condition of growing old, especially the condition resulting from the transitions and accumulations of the deleterious aging process. A 574 base pair mitochondrial fragment containing part of the cytochrome oxidase I gene was used to probe total DNA isolations from mouse cardiac tissue. A similar region in the rat mitochondrial genome has been shown to have minimal cross hybridization to genomic DNA (genDNA) sequences. Relative intensities of total cardiac DNA isolates were analyzed on a phosphoimaging unit and compared to standard mtDNA intensities to determine mtDNA concentrations. Since low intensity aerobic exercise is known to increase mitochondrial energy metabolism, the effects of chronic physical activity was examined to determine if increases in mitochondrial ATP production affect the cardiac mtDNA template.

HYPOTHESES

1. H0 : Increasing age will have no effect on concentration of mitochondrial DNA in mouse cardiac muscle tissue.

   H1 : Increasing age will result in an increasing concentration of mitochondrial DNA in mouse cardiac muscle tissue.

2. HO : Mitochondrial DNA concentration will not change in adult or senescent mouse cardiac tissue in response to chronic aerobic exercise.

   H1 : Mitochondrial DNA content will be altered in concentration in adult and senescent mouse cardiac muscle tissue in response to chronic aerobic exercise.

Chapter 1

Review of Literature

General Effects of Aging on Exercise Performance

There are several physiological changes associated with aging that affect physical performance. These changes ultimately lead to a decline in maximal exercise capacity and heart rate, elevated systolic blood pressure, increased ventricular wall thickness, declines in glucose tolerance and lipid metabolism, and decreased muscle and bone mass (Goldberg & Hagberg 1990). Some of the more prominent causes for the age related decline in functional capacity are disease, life-style habits, and physical inactivity. Atherosclerotic coronary heart disease is a major contributing factor to the decline in cardiac function in aged individuals who are predisposed to vascular complications of stroke and myocardial infarction (Lakatta 1987, Bierman 1985). Diabetes mellitus contributes to decreased glucose tolerance and ultimately affects many major organ systems of the body (Goldberg & Coon 1987). Life-style habits including overeating, alcohol consumption, and smoking also adversely affect physical performance in the elderly (Goldberg and Hagberg 1990). In addition physical inactivity and overeating contributes significantly to declines in cardiovascular performance, muscle mass and strength, glucose tolerance, high-density lipoprotein levels, as well as affecting triglyceride metabolism and body composition (Goldberg and Hagberg 1990).

Maximal oxygen uptake (VO_2 max) has been shown to decline with advancing age (Astrand 1958, Robinson 1938). These cross sectional studies reported an average decline in

VO_2 max of 10% per decade in sedentary individuals. However, highly trained masters athletes who had been rigorously screened for disease, displayed only a 5% reduction per decade in VO_2 max (Heath *et al*1981). In addition, Dehn & Bruce (1972) reported that the VO_2 max of active individuals declined less rapidly than sedentary men, suggesting that a physically active lifestyle may attenuate declines in exercise capacity.

A recent investigation by Ogawa *et al* (1992) concluded that decreases in stroke volume, heart rate and arteriovenous oxygen difference all contribute to the age related decline in VO_2 max. This group examined the relative contributions of age, training status, sex, and body composition to the age-related decline in VO_2 max. The decline in VO_2 max with age was shown to be primarily due to a lower maximal cardiac output. A decreased stroke volume was the main contributor to the lower cardiac output with a smaller portion due to a slower maximal heart rate. These findings are in agreement with previous results obtained by Port *et al* (1980). In addition Ogawa *et al* (1992) provided evidence that the effects of age and training on VO_2 max, maximal cardiac output, and stroke volume cannot be fully explained by differences in body fat. VO_2 max normalized to fat free mass declined with age. This method of reporting VO_2 max values is more appropriate since muscle mass (Tzankoff and Norris 1977) and blood flow to muscle (Kroese 1977) has been shown to decline with age. These results are in agreement with Fleg & Lakatta (1988) who also found VO_2 max normalized to muscle mass declined less rapidly in healthy older sedentary individuals. Ogawa *et al* (1992) also concluded endurance exercise training may attenuate age-related decreases in maximal heart rate and arteriovenous oxygen difference. In contrast, Rodeheffer *et al* (1984) found cardiac output during maximal exertion did not significantly

decline with age. This group found the characteristic decline in heart rate associated with age, but concluded that this decline was compensated for by an increased stroke volume, mainly due to increased left ventricular end-diastolic volume. However, Ogawa noted that subjects were not matched by sex or level of physical activity and that this may have had a significant effect on the left ventricular end-diastolic volume, stroke volume, and cardiac output data produced in the Rodeheffer study.

Cardiac Responses to Exercise

During exercise skeletal muscles increase their rate of metabolism in order to sustain an elevated work load. These elevated metabolic levels require greater oxygen delivery and the removal of metabolic byproducts through increased blood flow to the working muscles. Thus, in the exercising state, an acute central response (increased heart rate and stroke volume) is initiated.

Heart rate is controlled through intrinsic and extrinsic mechanisms. Intrinsic control is accomplished by the innate pacemaker ability of the sinoatrial node which has an inherent rhymicity of 70 to 80 beats per minute. Thus, in the absence of extrinsic input, the heart will spontaneously contract at an inherent rate. Extrinsic regulation is accomplished through nervous and hormonal stimulation which act to alter the inherent rhymicity. Neural influences originate in the cardiovascular center of the medulla and are transmitted through the sympathetic and parasympathetic branches of the autonomic nervous system. Sympathetic stimulation results in an increased heart rate while parasympathetic stimulation results in

bradycardia. Numerous peripheral receptors convey information to the cardiovascular center which in turn modifies cardiovascular response.

Epinephrine and norepinephrine, released from the adrenal medulla in response to exercise, mimic the sympathetic nervous response. The levels of these neurohormones released is relative to the amount of exercise performed. In trained individuals the sympathoadrenal response to a given workload is lower than in untrained individuals and is manifested as bradycardia coupled with a smaller rise in blood pressure during submaximal exercise. Sympathetic activity also affects blood flow redistribution, cardiac contractility, substrate mobilization, liver glycogenolysis, and adipose tissue lipolysis (Mckardle & Katch 1990). These β-adrenergic responses of the heart are diminished with age. In a study by Stratton *et al* (1992), age-related declines in heart rate, blood pressure, ejection fraction, and cardiac output responses to isoproterenol were observed. This group also noted intensive training did not increase cardiac responses in either young or old subjects.

In addition to elevated heart rate, increased cardiac output is achieved through significant increases in stroke volume during exercise. This is accomplished through enhanced diastolic filling and improved systolic emptying. Several vascular changes complement increased blood flow during exercise including redistribution of blood from visceral organs to working muscles, reduction in total peripheral resistance, and improved venous return (Mckardle & Katch 1991).

Training Effects on Cardiac Performance

Endurance training regimens lead to significant cardiac and cardiovascular adaptations. Repetitive exercise modifies both heart rate and stroke volume. Chronic aerobic training reduces heart rate at any given workload including resting conditions and could be a result of either reduced sympathetic stimulation or increased parasympathetic activity. This reduced heart rate necessarily requires an enhanced stroke volume to maintain cardiac output. Indeed with aerobic training significant increases in stroke volume have been amply documented (for review see Dowel 1983).

Older individuals exposed to an endurance training regimen significantly increase VO_2 max and undergo skeletal muscle adaptations similar to those reported in young individuals (Coggan *et al* 1992). In a comparative study by Douglas and O'Toole (1992) older ultraendurance athletes differed from their sedentary counterparts in having lower heart rates, larger left ventricular cavities, and higher ratios of early to atrial inflow velocities. These selected older athletes were exceptional, however, and allowances for cumulative training effects and genetic endowments must be given. In an earlier study by Heath *et al* (1981) VO_2 max of older endurance athletes was 60% higher than untrained middle-aged men following correction for body fat content. These two studies demonstrated an improved performance of older endurance athletes over their sedentary counterparts, however, exercise capacity was lower than that of young endurance trained athletes. The results of these investigations suggests that long term physical activity leads to enhanced exercise capacity in older individuals, and may blunt the age-related declines in exercise capacity. There is some indication that exercise adaptations in older individuals differs from that seen in young

individuals. For example, older individuals exposed to a 6 month program of low intensity training followed by 6 months of a higher intensity program resulted in significant increases in VO_2 max (Seals *et al* 1984), however, no improvement in left ventricular function was noted (Seals *et al* 1985). These results suggest that although increases in VO_2 max are possible in older individuals with training, gains are made through widening the arteriovenous oxygen difference (i.e. peripheral adaptations) and not by altering the cardiac output (i.e. central adaptations)(Hagberg 1987).

Metabolic Aspects of the Mammalian Heart

Mammalian cardiac tissue has the capacity to utilize several ATP producing pathways to satisfy energy requirements. Circulating fatty acids are the most important energy source for cardiac tissue providing approximately 60 to 70 % of the energy requirement (Neely & Morgan 1974) while endogenous myocardial fatty acids represent the alternative source (Saddick and Lopaschuk 1991). In fact, under conditions of low circulating fatty acids, endogenous myocardial fatty acid reserves may contribute up to 50% of the total energy requirement. Glucose is the other major substrate utilized by myocardial tissue and contributes 15 to 55 % of oxidative metabolism under fasting conditions (Opie 1969). The normal healthy heart will derive most of its energy requirement through β-oxidation of fatty acids supplemented with glucose oxidation and glycolytic pathways whose contributions are dependent on several factors. In a study by Saddick and Lopaschuk (1991) glycolytic rates were found to be twice as high as glucose oxidative rates in rat hearts perfused in the absence of fatty acids, while under high concentrations of fatty acids glycolitic rates were 13 times that of glucose oxidation. These results demonstrate that the metabolism of fatty acids inhibit

glucose oxidation to a much greater extent than the glycolytic pathways. In addition, this group confirmed that glucose can not be the sole myocardial energy source since even under high glucose/low fat conditions glucose utilization contributed only 40 to 50 % of the overall energy requirement.

β-oxidation of fatty acids occurs in the inner mitochondrial matrix. Long chain fatty acids cannot traverse the inner mitochondrial membrane necessitating a special transport process. Carnitine is an essential cofactor of this translocation process (Fritz *et al*1962). Through the actions of carnitine palmitoyl-transferase I and II (or carnitine acyltransferase), carnitine present on the cytosolic side of the membrane forms a carnitine ester with an activated long chain fatty acid, is transported through a translocase into the mitochondrial matrix, where ester formation is reversed and carnitine is returned to the cytosol (Stryer 1988). Thus the rate of β-oxidation is dependent on this sequence of events. Interestingly, Hansford and Castro (1982) noted significant declines in the activity of carnitine acyltransferase in senescent rat heart, and suggested an impairment of the aged heart to metabolize long chain fatty acids. In addition, rates of myocardial palmitate oxidation were significantly reduced in hypertrophied rat hearts compared with controls during low work conditions, and was attributed to reduced levels of myocardial carnitine (Allard *et al* 1994).

Fleg (1986) reviewed several cross sectional studies using echocardiography which have demonstrated a progressive increase in left ventricular wall thickness with increasing age in human subjects. This may be a result of stiffening of the arterial wall which occurs with aging, leading to increased afterload on the heart (Goldberg and Hagberg 1990). These results suggest that the normal aging process of the heart may lead to metabolic alterations,

specifically declines in the ability to oxidize fatty acids, leading to an increased reliance on glucose and glycolytic pathways for energy production.

Mitochondrial Function and Age

There have been several reports suggesting that a decrease in respiratory capacity with senescence is associated with decreases in mitochondrial volume and aerobic enzyme activity. The bulk of evidence implicating mitochondrial declines with increasing senescence stem from biochemical studies. Abu-erreish and Sanadi (1978) reported that the activity of rat myocardial mitochondrial cytochromes decreased in 26 to 29 month old rats compared with 5 and 15 month old animals. This group further recognized that, since cytochromes are present in relatively constant proportions in mitochondria, the decrease in all the cytochromes with age is consistent with the concept of a decrease in the content of respiratory assemblies. They suggested these age-related declines may be due to altered synthesis, degradation, or malfunction of cytochrome assemblies in the mitochondrial membrane. Hansford and Castro (1982) reported significant decreases in mitochondrial enzymes citrate synthase, NAD-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase in rat skeletal and heart tissues while attempting to define potential tricarboxylate cycle activity (TCA) and its response to aging. The relative decreases found in cardiac tissue of these enzymes from 6 to 24 month old rats 18%, 16%, and 24% respectively. During this study carnitine acetyl transferase activity was also investigated and found to decrease by 51% in the older animals compared to their adult counterparts. These observations not only describe age-related compromises in mitochondrial oxidative enzymes but also indicates an impairment of the aged heart to metabolize long chain fatty acids due to poor substrate transport mechanisms. Since these

molecules are a preferred fuel for cardiac metabolism this finding of substrate deprivation may also contribute to the overall decrease in cardiac performance with age. Takasawa *et al* (1993) also reported significant age-related decreases in activities of complex I and complex IV of rat heart mitochondria. Decreases of 31% and 22% in these complexes respectively occurred in 100 week old rats compared to 7 week animals. Morphometric studies by Herbener (1976) reported declines of 16% occurred in 43 to 44 month mice compared to 8 month in mitochondrial volume density in the aging mouse heart which lends support to these decreased enzyme activities. This evidence is contrasted by findings of Starnes *et al* (1981) who reported no age-related declines in oxidative phosphorylation catalyzed by mitochondria from non-perfused rat hearts. It should be noted, however, that this study did observe a significant decrease in the capacity of the senescent heart to adapt to elevated perfused workloads.

The results of the above studies provide evidence that respiratory capacity declines in senescent tissue and that these declines are associated with decreased mitochondrial volume and enzyme activities. These reductions in oxidative capacity may be a result of several factors. Some suggestions include a decrease in mtDNA transcription or translation efficiencies, possibly due to an increase protein turnover rate of proteins and/or factors involved in these processes; decreases in mtDNA template availability resulting in lower amounts of RNA products; and accumulations of alterations (mutations/deletions) in mtDNA templates leading to dysfunctional products. In each case the amount of mitochondrial DNA template present is essential to the explanation. The coordinated expression of mitochondrial and nuclear DNA transcripts is essential to produce viable, functioning mitochondria (Hood

1990). However, specific inhibition of mitochondrial translation does not affect nuclear mitochondrial transcripts (Williams & Harlan 1987). In addition it has been demonstrated that elevated mitochondrial protein synthesis induced by chronic stimulation is coupled with elevated concentrations of mtDNA in rabbit skeletal muscle (Williams *et al* 1987). These results led to suggestions that mitochondrial adaptation to elevated energy demands may be regulated at the level of template availability (Gadelata *et al* 1992).

Characteristics of The Mammalian Mitochondrial Genome

The mammalian mitochondrial genome has been characterized as a closed, circular, double stranded DNA structure consisting of approximately 16 kilobase pairs. This is small compared to the nuclear genome from which the bulk of mitochondrial proteins are encoded and subsequently transported into mitochondria through complex transport mechanisms. Mitochondrially encoded subunits are nevertheless essential for the formation of functionally intact respiratory complexes. The assembly of a functioning mitochondrial energy transducing system depends on the coordinated expression of the nuclear and mitochondrial genetic systems.

Mammalian mitochondrial DNA is extremely compact, saturated by coding regions save a small region thought to be involved with regulation. The two mitochondrial DNA strands (heavy and light) are transcribed as polycistronic RNA molecules from promoters located near the D-loop region resulting in complete symmetrical transcription of the genome. Another interesting fact is that there are two overlapping H strand transcription units necessitating the need for precise RNA processing apparatus. Most genes are transcribed

from the heavy strand and are generally blunt ligated. Transcribed genes may lack a complete termination sequence, a problem which is overcome by post transcriptional polyadenylation of mRNA's (for review see Attardi and Schatz 1988).

Several mammalian mitochondrial genomes have been sequenced and found to encode seven subunits of complex I (NADH coenzyme Q oxidoreductase), one subunit of complex III (ubiquinol-cytochrome c oxidoreductase), three subunits of complex IV (cytochrome c oxidase), and two subunits of complex V (ATP synthase). In addition components of the mitochondrial translation system are also encoded in mtDNA including 22 tRNA genes as well as genes encoding the 16S and 12S mitochondrial ribosomal RNA's (Anderson 1981).

Biogenesis of mitochondria involves the formation of mitomembranes and the differentiation of organelles for oxidative phosphorylation (Attardi & Schatz 1988). The latter events require input from both mitochondrial and nuclear genomes. It is generally accepted that biological membranes do not form de novo and therefore mitochondrial membranes are believed to arise from pre-existing material. Mitochondria are replenished through a self fission process similar to that seen in prokaryotes. Mitochondrial DNA replication occurs in the late S and G2 phases of the cell while growth of mitochondrial membranes continues throughout the cycle. This indicates little temporal coupling of the growth of the organelles and mtDNA replication (review Attardi and Schatz 1988).

The Question of Template Availability

Although there is wide acceptance that respiratory capacity declines with age due to the factors outlined above there is still debate over the content changes of mitochondrial DNA with senescence. Massie *et al* (1975), demonstrated a significant decrease in mitochondrial DNA during aging in *Drosophila melanogaster*, while nuclear DNA remained constant. Since the buoyant densities of mtDNA and nuclear DNA's differ, separation was achieved through rate zonal centrifugation in a cesium chloride (CsCl) gradient. Their results led them to support the proposal that destruction of mitochondria occurs with senescence. In a later study, Stocco *et al* (1978) reported significant decreases in liver mitochondrial DNA and protein in 24 month old Fisher 344 rats compared to 12 month old animals. In this investigation mitochondria were first purified by rate zonal centrifugation followed by purification and measurement of mtDNA by a modification of the diphenylamine reaction. Since the mtDNA decreases were not coupled to losses of cytochrome oxidase or lipoamide dehydrogenase activities (mitochondrial specific enzymes) they supported the same conclusions as Massie *et al* (1975). Through similar mitochondrial isolation and purification procedures Takasawa *et al* (1993) found significant decreases in rat heart tissue (25 month compared with 7 week old animals), however no change in rat liver mtDNA was found in contrast to Stocco et al (1978) observations. Measurement of mtDNA content in the Takasawa *et al* (1993) study was achieved through high performance liquid chromatography and mass spectrometry analysis in which quantification of each nucleoside was performed. Asano *et al* (1992) found that rat liver mtDNA did in fact decrease with increasing age. This group utilized non-radioactive mitochondrial specific probes to detect mtDNA from total

DNA extracted from the tissue. The different results obtained in each study may be a result of the different methodology used to estimate mitochondrial DNA concentrations. The use of rate zonal centrifugation to isolate mitochondria as a preliminary step to mtDNA content analysis may lead to selective loss of mitochondria susceptible to rupture. Methodological differences aside these studies generally agree that mtDNA content decreases or is unchanged in various tissues with increasing senescence.

These studies are contrasted by investigations done by Gadaleta *et al* (1990, 1992). Initially they found that with increasing age (using a rat model system) the level of mitochondrial specific RNA products were significantly reduced and that this reduction could be reversed by treatment with acetyl-L-carnitine. Acetyl-L-carnitine is involved in a number of major biochemical pathways and acts to stimulate energy metabolism in the mitochondria. It is also an essential component in the transportation of long chain fatty acids across the inner mitochondrial membrane to the site of β-oxidation. Since treatment with this compound had no effect on mtRNA in adult rats the decrease in RNA was attributed to a decreased mtDNA transcription rate in senescent individuals. This was confirmed by Fernandez-Silva *et al* (1991) who found mtRNA synthesis to be reduced in mitochondria isolated from senescent rat brain and attributed the decrease to a reduced mtDNA transcription rate. Investigations by Williams *et al* (1986; 1987) found mtRNA content to be directly proportional to the mtDNA copy number in young animals following nerve stimulation suggesting control at the level of template availability. From these studies they reasoned that this situation may be caused by a reduced mtDNA template availability or to an alteration in mtDNA structure, which an increase in ATP production could attenuate by supplying sufficient energy for production of

more supercoiled mtDNA molecules (for review see Gadaleta *et al* 1990). However, subsequent research by this group has shown the mtDNA of aging rats increases in heart, skeletal muscle, and brain tissues contrary to earlier investigations (Gadaleta *et al* 1992). These latter findings have led to their conclusion that decreased mtDNA transcription rates are not due to a reduction in mtDNA copy number with senescence.

If the loss of template is responsible for decreases in mtRNA, and subsequently mitochondrial protein, then logically we would expect to see a decrease in mitochondrial DNA. Thus the increase in mitochondrial template availability with age observed by Gadaleta *et al* (1992) may be a compensatory response to decreases elsewhere along the DNA to protein pathway or possibly to substrate restriction by dysfunctional mitochondrial membrane transport mechanisms. Indeed the reversal of mtRNA declines by acetyl-L-carnitine observed by Gadaleta *et al* (1990) coupled with significant declines in carnitine acetyl transferase activity observed by Hansford and Castro (1982) lends support for the latter explanation. Other possible mechanisms for compromises of the DNA to protein pathway include increased rates of degradation of proteins involved in transcription/translation of mitochondrial products; decreased transcription/translation efficiency of mitochondrial products; or accumulations of mitochondrial DNA mutations leading to dysfunctional gene products and defective mitochondria. However, the latter explanation does not appear plausible since researchers have found mitochondrial DNA mutations only account for < 1% of the total mitochondrial DNA present (Gadaleta *et al* 1992, Corral-Debrinski *et al* 1992, Simonetti *et al* 1992, Arnheim and Cortopassi 1992).

In summary, cardiac function decreases with age contributing to the lower VO_2 max values observed in older individuals. The decline in cardiac function can be attributed mainly to decreased heart rate and stroke volume. At the cellular level cardiac mitochondrial oxidative capacity is reduced and may be a consequence of reduced transcription of mtDNA to form mitochondrial specific proteins. There is evidence that mitochondrial transcription is controlled at the level of template availability and that an adaptive response to chronic stimulation leads to an increase in mtDNA. However, there are conflicting reports as to the changes in cardiac mtDNA content with age. Gadaleta *et al* (1992) observed an increase in mtDNA in aged rat hearts and suggested this may reflect a compensatory response to a compromised transcription process in cardiac mitochondria. However, these increases in mtDNA with age require confirmation. In addition, chronic aerobic exercise leads to adaptive responses in both adult and senescent hearts and the extent to which these responses are linked to mtDNA content has not been examined. The purpose of this study was to examine the effects of aging and physical activity on heart mtDNA template availability.

Chapter 2

METHODS

Animals and Activity

Female C57/BL6 mice aged 4, 8, and 24 months were used in this study. This strain, chosen for its inherent activity and bred for pathogen-free studies, is mature at 3 to 4 months of age with an average life span of approximately 30 months. Mice were obtained from Charles River Breeding Laboratories (Quebec) and upon arrival were maintained on a 12 hour light dark cycle and have food (Purina Rodent Lab Diet 5001, Illinois, USA) and water *ad libitum*. The mature (8 month) and senescent (24 month) animals were randomly assigned to either control or exercised groups. Control animals were housed (n = 3 to 8) in conventional cages. The mature exercised animals were also group housed but had access to a freely rotating hamster wheel (16 cm diameter) 3 hours per day for 9 weeks from 5 months of age. The senescent exercised animals were individually housed and had access to a freely rotating hamster wheel (16 cm diameter) for 24 hours per day for 36 weeks from 15 months of age.

A magnetic switch was attached to each exercise wheel and digital data were relayed continuously to a computer. A computer program was developed to calculate the number of exercise bouts, the average velocity and distance run of each exercise bout and results were expressed on a per hour basis. A delay of more than 1 minute between subsequent revolutions of the exercise wheel represented the end of the exercise bout. The total distance

run was calculated and expressed per 24 hour period. Any animal that did not run on average more than 1.2 km per day was excluded from the study. Animals were removed from the wheels 48 hours prior to sacrifice. Since the absolute workloads were different and the relative intensity of each exercise bout for each age group was not determined, it is impossible to compare between exercise groups. The intention of the study was not to compare between the exercised groups of different ages but to see how each individual group responded to the increased metabolic rates associated with chronic exposure to the exercise wheels. Animals were anesthetized using sodium pentabarbitol (100 mg/kg body weight). The heart was removed, washed in saline, frozen in liquid nitrogen, and stored at -80 °C. Citrate synthase was measured as a marker for oxidative capacity. Briefly, 20 - 30 mg of tissue sample were homogenized in 50 volumes of buffer (50 mM Tris HCl pH 7.6, 1 mM EDTA, 0.1 % Triton X-100) followed by brief sonication (repeat homogenization and sonication). The resulting homogenate was centrifuged at 12000 rpm (Beckman benchtop centrifuge) for 10 minutes at 4 °C. Citrate synthase was assayed in 0.2 mM Acetyl CoA, 0.2 mM DTNB, 50 mM Tris-HCl pH 8.1 at 25 °C and the absorbance was recorded at 412 nm.

DNA Isolation Procedure

Whole hearts (100 to 150 mg) were briefly washed in 70% EtOH, blot dried, and weighed. The sample was then suspended in 4 mls of lysis Buffer (2 % Triton X-100, 100 mM Tris-HCl, pH 7.5), 0.5 ml Proteinase K (20 mg/ml) and 0.5 ml 2 M Guanidine HCl. The samples were then incubated at 55 to 60 °C with periodic mild vortexing for 30 seconds every 30 minutes for 3 to 4 hours. Samples were then left overnight at 55 to 60 °C. Large cellular

debris was removed by centrifugation at 10,000 rpm for 30 minutes in a Sorval GS25 rotor at 4 °C. The supernatant was then treated with 20 µL of 1 mg/ml RNase A and incubated for 30 minutes at 37 °C. DNA was purified from the supernatant using Qiagen® Cell Culture DNA isolation kit. The resulting purified DNA eluant was precipitated with 0.7 volumes of isopropanol and centrifuged at 4 °C in a Sorval GS25 rotor at 10,000 rpm for 30 minutes. The DNA pellet was then washed with 70% EtOH, air dried, and redissolved in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). This procedure resulted in good clean DNA which was readily digested by restriction enzymes (Figure 1). Quantification of total DNA (genomic and mitochondrial) was obtained by absorbance at 260 nm. A standard curve was generated by measuring A260 of 0.5, 1, 2, 4, 8, and 10 µL of λ DNA-Hind III (purchased from Gibco/BRL®; known concentration of 0.709 ug/µL) in duplicate (Figure 2). Each DNA sample was measured in duplicate and corrected to the standard curve.

Southern Blotting

In order to demonstrate the low cross reactivity with nuclear sequences and high specificity to mitochondrial sequences of the selected probe, a southern blot analysis was performed (Figure 3). Several known quantities of Sst1 linearized control mitochondrial DNA and unlabelled 958 bp mitochondrial specific probe (concentrations of 10, 5, 1, 0.1, and 0.01 ng) were electrophoresed against known amounts of total mouse DNA isolated from mouse skeletal muscle (total mouse DNA was digested with Sst1, concentrations of 2, 1, 0.2 ugs) through a 1% agarose gel at 15 V overnight. The gel was then washed as follows; 20 min. 0.25 M HCl, 20 min. 0.4M NaOH/1.5M NaCl, 20 min. 1M Tris/ 1.5M NaCl, and 5 min.

10x SSC. The gel was then transferred to Zeta probe nylon membrane using the standard capillary transfer setup with 10x SSC as the transfer buffer. Following overnight transfer the membrane was then washed in 2x SSC and crosslinked in a Stratalinker on Autocrosslink cycle. The membrane was then stored in 1% SDS solution in a sealed bag for later hybridization with a ^{32}P labeled 958 bp mitochondrial specific probe (see hybridization procedures this section).

Slot Blotting Procedures

Mitochondrial DNA content in mouse cardiac tissue (per milligram wet tissue and per μg nuclear DNA) was quantified using a slot blot apparatus. Quantification was achieved by comparing the relative intensities of isolated sample mouse cardiac DNA to intensities generated from standard mitochondrial DNA quantities present on the same blot. Relative intensities were measured using a BioRad phosphoimaging unit and related software. Known amounts of Sst1 digested total genomic DNA samples (250 ng) and linearized control mitochondrial DNA (5, 1, 0.1, 0.01 ng) were combined with 1M NaOH (final concentration of 0.4M) and 0.5M EDTA (final concentration of 10 mM) in 0.5 ml polypropylene Eppendorf tubes. Tubes were heated to 100 °C for 10 minutes on a Biometra® PCR block then rapidly cooled on ice for 5 to 10 minutes. Following brief centrifugation of tubes standard/sample DNA was transferred to presoaked nylon membrane through a vacuum assisted slot blot apparatus. Each slot was then rinsed with 200 μL of 0.4M NaOH, 10 mM EDTA. The membrane was then briefly rinsed in 2x SSC and crosslinked in Stratagene® Stratalinker on autocrosslink cycle. Membranes were stored in sealed bags containing 1% SDS for later

hybridization with a ^{32}P labeled 574 bp mitochondrial specific probe (see hybridization procedures this section and Figure 4).

Mitochondrial DNA Standard

A plasmid (pSP64 Promega®, see Figure 5) containing the entire mouse mitochondrial genome has been obtained from Dr. W. Hauswirth at the University of Florida, Gainsville. Sst 1 cleaves the 16295 bp mitochondrial genome at nucleotide 9048 and pSP64 at nucleotide 50 located in the polylinker region (note that SstI is an isoschizomer of Sac I depicted in Figure 5). The mitochondrial clone was received in lyophilized form and was resuspended in TE buffer. The clone was transformed into competent DH5 alpha cells, plated on LB-amp plates and incubated at 37 °C overnight. Single colonies were inoculated in LB-amp broth and incubated at 37 °C overnight with shaking. Plasmid DNA was extracted from overnight broths using a Magic Miniprep Kit (Promega®). Verification of the cloned mitochondrial insert was accomplished by restriction fragment length polymorphism (RFLP) using various restriction enzymes (Figure 6). This purified mitochondrial DNA was the standard used in comparing quantities of mitochondrial DNA from sample genomic DNA extraction's on hybridization blots (see Figure 4).

Mitochondrial DNA Specific Probe

Utilizing the cloned mouse mitochondrial DNA described in the previous section a 958 base pair fragment was obtained through a Polymerase Chain Reaction ("PCR", see Figure 7) using oligonucleotide primers located at H5942 and L4984 ('H' and 'L' denote

heavy or light strand orientation and numbers refer to nucleotide positions of mouse mitochondrial DNA published by Bibb *et al,* 1981). This fragment contains mitochondrial tRNA's Trp, Ala, Asn, Cys, and Tyr, as well as 617 bases from the 3' end of the cytochrome oxidase I gene. Primer sequences and orientation with respect to the mouse mitochondrial genome is as depicted in Figure10. The PCR reaction solution contained 1 µL Vent® Polymerase (New England Biolabs), 5 µL 10x Vent reaction buffer, 1 µL 25mM dNTP's (6.25 mM each of dATP, dGTP, dCTP, dTTP), 1 µL Sst I linearized mouse mitochondrial DNA (1 to 20 ng), 1 µL each of primers (500 to 600 ng each), and 40 µL ddH_2O for a 50 µL reaction. Reaction conditions consisted of 35 cycles of 95 °C for 30 seconds (denaturization), 52 °C for 50 seconds (annealing), and 72 °C for 100 seconds (polymerization). These reaction conditions resulted in amplification of the target sequence with no evidence of mispriming (i.e. nonspecfic bands; Figure 11). Following PCR the amplified 958 base pair mtDNA fragment was electrophoresed through a 1 % agarose gel. The fragment was isolated from the gel by low speed centrifugation through sterile glass wool. The fragment was labelled using the Random Priming System (New England Biolabs®). Unincorporated nucleotides were separated from the labelled fragments by passing the reaction through a Sephadex G50 column (approximately 2 ml). The labelled fragment was subsequently tested for potential cross hybridization to nuclear sequences (see Southern Blotting section; Figure 3). Although low cross reactivity was demonstrated at concentrations of 200 ng, to ensure higher specificity the tRNA sequences were eliminated from the fragment with EcoR V digestion (Figure 12) resulting in a 574 base pair mtDNA probe. The probe was subsequently used for slot blot analysis of mitochondrial DNA sequences. A similar region in the rat mitochondrial

DNA (nucleotides 4253 through 5942 as published by Gadaleta *et al* 1989) was shown to have minimal cross hybridization to genomic sequences (tRNA's inclusive)(Asano *et al* 1992). This region in the mouse mitochondrial genome was highly conserved compared to the rat sequence (87 % homology within the cytochrome oxidase I coding sequence) and was therefore selected as the probe of choice.

Hybridization Procedures

Blots were prehybridized for one hour at 65 °C (hybridization solution: 1 mM EDTA, 0.25 M NaH_2PO_4 pH 7.2, 7% SDS). The prehybridization solution was discarded and replaced with fresh hybridization solution warmed to 65 °C containing ^{32}P labeled denatured probe. Probes were denatured by boiling for 10 minutes followed by cooling on ice. Following overnight hybridization blots were washed (2 x 30 min. 1 mM EDTA, 40 mM NaH_2PO_4 pH 7.2, 5% SDS; 2 x 30 min. 1 mM EDTA, 40 mM NaH_2PO_4 pH 7.2, 1% SDS) at 65 °C. Blots were sealed in plastic bags and exposed to a phosphoimaging screen.

Data Analysis

All results are reported as the mean ± SE. All comparisons with respect to aging alone were analyzed by ANOVA for significance. If significance was indicated, a student-Newman-Keuls post-hoc analysis was carried out. Similar changes between control and voluntary exercised groups were analyzed using unpaired t-tests to identify significant differences.

Chapter 3

RESULTS

Body Weight, Heart Data and Wheel Running Activities

Animal body weights were not significantly affected following voluntary exercise in either the 8 or 24 month age groups. Average animal weights for these animals were 22.1, 24.1, and 30.5 g for 4, 8, and 24 month mice, respectively (Table 1). Average absolute heart weights were 77, 108, and 133 for 4, 8, and 24 month sedentary mice respectively (Table 1). The absolute heart weight was found to significantly increase with age (Table 1) with a 41% and 74% increase in weight for 8 and 24 month animals respectively when compared to 4 month mice. Although exposure to the activity wheel resulted in increases in absolute heart weights for the 8 (117 mg) and 24 (145 mg) month old animals, only the senescent exercised animals heart weights were significantly different from their age-matched controls (Table 1). These data translated into heart weight to body weight ratios for 8 and 24 month old animals that were significantly increased from the 4 month old animal values (Table 1). 8 and 24 month voluntary wheel trained groups however, displayed no change in heart weight to body weight ratios. The 8 month old animals had limited access to the exercise wheels so that their total distance run per day (1.9 km/day) was similar to the 24 month old animals (2.1 km/day) (Table 1). Although the number of runs per hour did not differ with respect to age, average run time and velocity was lower in 24 month (8 min/run, 10 m/min) compared to adult animals (11 min/run, 15 m/min). Citrate Synthase activities were determined on all age and

treatment groups as a marker to assess alterations to the mitochondrial component in mouse cardiac tissue. Although a declining trend was evident no significant change was noted between 4, 8, and 24 month sedentary control values (Table 1). 8 month voluntary exercised mice exhibited significantly increased citrate synthase activities compared to sedentary controls ($P \leq 0.05$), while no difference occurred between control and wheel trained groups in the 24 month animals (Table 1).

Mitochondrial DNA Content Changes with Age

Mitochondrial DNA content per mg of tissue significantly increased with increasing age (Figure 8). A 19% and 79% increase occurred in 8 month and 24 month animals respectively compared to 4 month mice. No significant change was found in comparisons of control and voluntary exercised groups in 8 or 24 month mice (Figure 8). However, even though significance was not found ($P \leq 0.08$), 8 month voluntary exercised mice had an average of 48% higher mtDNA content than sedentary controls.

Alterations in mtDNA:nuDNA Ratio

The mtDNA to nuDNA content significantly increased with age. 8 and 24 month control groups were 46.5% and 108% higher than 4 month counterparts (Figure 9). In addition, the 8 month wheel trained ratio (1.86 ± 0.23) was significantly higher than corresponding age matched controls, while no change was found between 24 month control and wheel trained groups (Figure 9).

Chapter 4

DISCUSSION

The results of the present study indicate that mouse cardiac mtDNA template increased with age. This was apparent by noting mitochondrial DNA content per milligram of wet tissue increased with age, as did the mitochondrial DNA to nuclear DNA ratio. These findings are in agreement with Gadaleta *et al* (1992) investigation which reported increases in mtDNA content in rat liver, heart, and cerebral hemisphere's. In contrast a significant decrease in mtDNA with age was reported by Takasawa *et al,* 1993. The latter investigation utilized differential centrifugation to isolate whole mitochondria prior to isolation of mitochondrial DNA. Thus methodology may have contributed to the observed differences since the present study and Gadaleta et al (1992) utilized probe hybridizations on total extracted DNA from nascent wet cardiac tissue. A hypothetical explanation could be that mitochondria from older animals have undergone age-related degenerative changes in the outer membrane resulting in organelles susceptible to rupture. This situation could result in potential rupture of mitochondria of marginal viability during the differential centrifugation isolation procedure. Evidence for this hypothesis may be provided by investigations displaying the increased susceptibility of mitochondria to oxygen free radical attack. Univalent reduction of oxygen results in a series of cytotoxic oxygen species such as superoxide, hydrogen peroxides, and hydroxyl radicals which are products of normal metabolism (Chance *et al,* 1979). These highly reactive species can cause a wide spectrum of

cell damage including lipid peroxidation, inactivation of enzymes, and damage of DNA (Slater *et al,* 1987; Ritcher, 1992).

Other studies investigating mitochondrial DNA content alterations with age were carried out by Stocco *et al* (1978) and Asano *et al* (1992) in rat liver, who both found significant decreases in mtDNA template in this tissue. These results were contrasted by Takasawa *et al* (1993) who found no change in rat liver mtDNA template concentration. These studies reflect the possibility that different tissues may respond differently, with respect to mitochondrial components, during aging.

It is our contention that by using our method mitochondria of questionable viability are not biased and a better representation of total cellular mitochondrial DNA is reflected. The observation of increasing mtDNA with age may be best explained by a "compensatory response" as suggested by Gadaleta *et al*(1992). Studies by Williams laboratory have shown that mtRNA content was directly proportional to mtDNA copy number while studying mtRNA changes in response to nerve stimulation in rabbit skeletal muscle (Williams *et al* 1986, Williams *et al* 1987). These observations support control at the level of template availability. Thus compromises to the energy transducing pathway due to aging responses in cardiac tissue may have led to higher replicative rates of mtDNA to induce elevated mitochondrial specific proteins. It would appear that any compensatory response (i.e. elevating mtDNA template) is insufficient to completely counteract the negative affects of aging on oxidative phosphorylation resulting in the steady template increase with age.

Although our results have indicated a significant increase in mtDNA template with increasing age in this model, we failed to see any significant decreases in citrate synthase

activities with age as described by previous literature. For example Hansford & Castro (1982) noted an 18% decline in cardiac citrate synthase activity from 6 to 24 month Wistar rats. In addition Abu-erriesh & Sanadi (1978) reported 27% declines in cytochrome oxidase activities from 7 to 29 month old Sprague Dawley rats. However, our results do show a declining trend between 4 and 24 month mice of approximately 10%, and may have displayed significance with a higher number of animals tested. Another possible explanation for not identifying declines in oxidative capacity may be due to discrepancies in response to aging of activities of enzymes used to measure oxidative capacity (citrate synthase vs. cytochrome c oxidase). Evidence for the latter possibility stems from the fact that citrate synthase is entirely nuclear encoded and compromises to the mitochondrial genetic system may not have been manifested in this enzyme. This has been demonstrated by Williams and Harlan (1987) investigation in rabbit skeletal muscle. In their study the inhibition of mitochondrial ribosomes by chloramphenicol administration abolished the normal activity-induced increases in cytochrome c oxidase (which contains mitochondrial and nuclear encoded subunits) seen with functional mitochondrial ribosomes. However, activity-induced increases in citrate synthase were not affected. In the present investigation we did not observe a significant decline in citrate synthase activity in response to the aging condition and therefore it is possible to apply a similar logic. In light of our results that mtDNA template and mtDNA to nuclear DNA ratio increase steadily with increasing age, and assuming these increases reflect compensatory measures, it appears that the adaptive pressure may be occurring within the mitochondrial genetic system and not the nuclear genetic system. The steady state activity of citrate synthase would be expected to remain relatively unchanged under this assumption.

Access to voluntary exercise resulted in significant increases in mtDNA template for 8 month animals, whereas 24 month animals remained essentially unchanged. 8 month voluntary wheel trained animals exhibited higher mtDNA to nuclear DNA ratios and a 48% higher mtDNA content than sedentary controls. Since 8 month animals exhibited higher rates and distances on the wheels this data may be indicative of a training adaptation. In support of this conclusion citrate synthase activities in these animals were found to be significantly higher than sedentary counterparts whereas 24 month trained animals had similar citrate synthase activities as age matched controls despite the longer duration training regime (36 vs. 9 weeks). These conclusions are challenged by several reports that endurance training does not lead to improvements in oxidative capacity of cardiac tissue in young aged animals (Oscai *et al* 1971, Rockstein *et al* 1978, Tibbits *et al* 1978, Tibbits *et al* 1981, Salminen and Vihko 1980, 1981, 1983).

Our observation that voluntary exercise did not affect citrate synthase activities in 24 month rat cardiac tissue is contrasted by studies performed by Starnes *et al*(1983) who reported significant increases (12%) in senescent (24 month) versus mature (9 month) Sprague-Dawley rat cardiac cytochrome c concentrations with forced treadmill training (approximately 22 m/min for 35 min each day for 16 weeks). These discrepant results are possibly due to insufficient training stimulus in the current study (average 8.9 m/min vs. 22 m/min peak treadmill velocities) or species differences. Similar decreased running activities in aged C57bl6 mice have been previously reported by Samorajski and Rolsten (1975). Although some previous literature suggest that exercise does not induce cardiac oxidative improvements some investigations have utilized similar low intensity training. Reznick et al

(1982) reported no change in mouse cardiac oxidative markers following a 5 week forced treadmill training of 27 month old animals exposed to 30 min/day at 3.5 m/min (approximately 0.1 km/day total distance). These distances and velocities are significantly lower than reported in this study (8.6 m/min, 2.1 km/day). In contrast German et al (1986) reported significant increases in oxygen comsumption and skeletal muscle enzyme activities in 20 month old C57bl6 male mice exposed to forced treadmill training (15m/min, 30 min/day, 5 days/week, 8 weeks). Average total distance run by these mice were 0.45 km/day. Increases of 20% in SDH activity of the vastus lateralis muscle was interpreted by this group as a training effect. Comparison to our reported running data indicate animals in this study were subjected to a more intensive exercise program.

Voluntary exercise has previously been found to lead to training adaptations in rodents indicating the protocol for exercise in this study was justified. For example Sexton (1995) reported that voluntary exercise in 4 month Sprague-Dawley rats led to increases in citrate synthase activities in recruited skeletal muscle. An earlier study by Goodrick (1980) indicated that long term voluntary exercise in Wistar rats led to significant increases in overall metabolic rates compared to controls beginning at the 12 month age level.

There are several possible mechanisms which may contribute to the observed increases in mtDNA content in aging mouse cardiac tissue. Assuming that control of mitochondrial derived products is regulated at the level of template availability, compromises to the transcriptional or translational mitochondrial systems, or changes in the metabolic profile of cardiac mitochondria, may contribute to the observed condition. Mitochondrial transcription has been reported to be decreased in aging rat cardiac tissue despite significant

increases in mitochondrial DNA content (Gadaleta *et al* 1990, 1992). In addition decreased rates of mitochondrial protein synthesis have been observed in senescent mouse hearts (Geary *et al* 1972). There has been some indication that cardiac mitochondria have a decreased ability to oxidize fatty acids (Hansford & Castro 1982) which has been attributed to low myocardial carnitine levels (Allard *et al* 1994, Gadaleta *et al* 1992). Thus it can be inferred that cardiac mitochondrial template increases with age may be a consequence of several age-related phenomenon.

CONCLUSIONS

Results of this investigation have demonstrated that mitochondrial DNA template availability increases in mouse cardiac tissue with senescence in agreement with Gadaleta et al conclusions (1992). This is reflected in increases in mitochondrial DNA content per milligram of cardiac tissue and mitochondrial DNA relative to nuclear DNA ratios. Oxidative capacity, as determined by citrate synthase activity, remained essentially unchanged in the three age groups studied. This may be indicative of sufficient compensatory effects brought about by increased mitochondrial template or reflects the stability of nuclear encoded mitochondrial proteins with increasing age.

Voluntary exercise was utilized as a means of elevating mitochondrial energy metabolism to test if increased mitochondrial ATP production affected mitochondrial DNA template levels. 8 month old mice responded positively displaying increased mitochondrial DNA to nuclear DNA ratios and elevated citrate synthase activities. Although widely

disputed in previous literature this indicates the voluntary activity levels of these mice was successful in inducing a cardiac training effect. Thus increasing demand on the mitochondrial system did result in elevated template levels in 8 month animals confirming regulation at the level of template availability in mouse cardiac tissue. The 24 month animals did not display these results, most likely due to low voluntary wheel activities, but still displayed elevated mitochondrial DNA content and mtDNA to nuDNA ratios compared to 8 month sedentary animals. Even so we were unable to definitively show that elevated cellular metabolism resulted in increased template benefiting the oxidative capacity of the 24 month heart.

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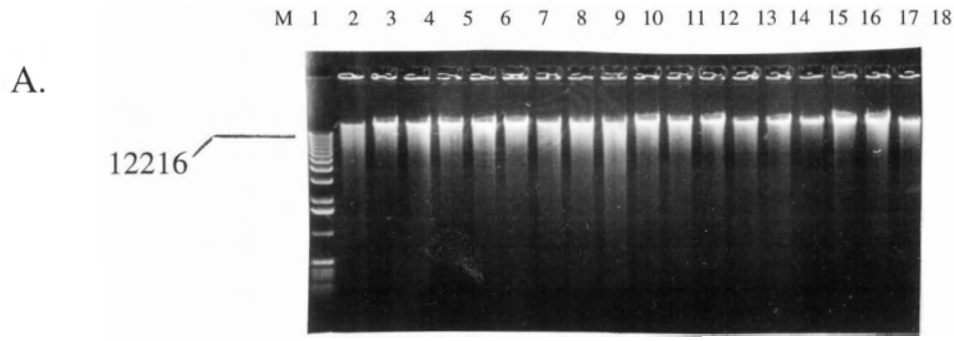
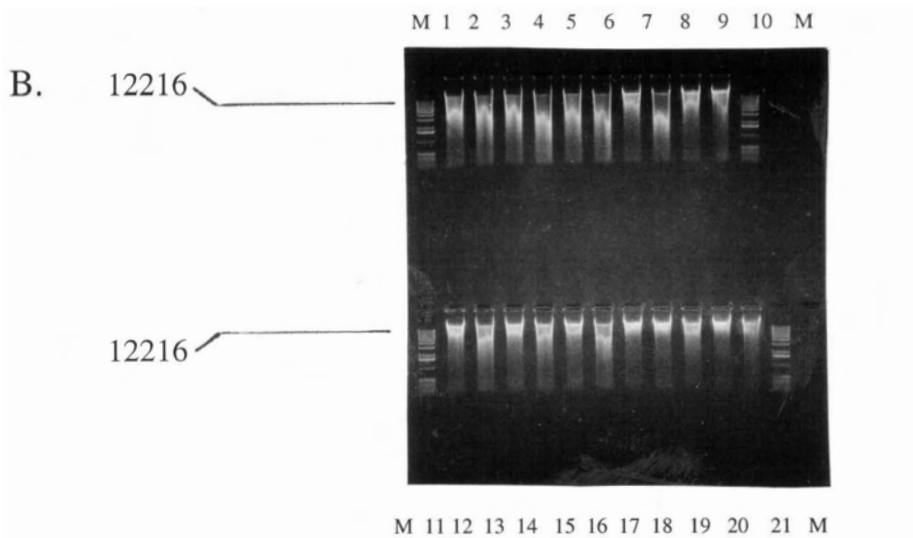
APPENDIX I

COMPILATION OF FIGURES

Figure 1: A) Total genomic DNA extracted from mouse skeletal muscle using Qiagen® DNA extraction kit. Lanes 1 - 6: 16 week control quadriceps; lanes 7 - 12: 8 month control quadriceps; lanes 13 - 18: 12 month control quadriceps. Each lane represents approximately 0.25 µg. (B) Total genomic DNA extracted from whole mouse hearts using Qiagen® DNA extraction kit. Lanes 1 - 6: 4 month control; lanes 7 - 12: 8 month control; lanes 13 - 18: 8 month voluntary exercised; lanes 19 - 21: 12 month control. Each lane represents approximately 1 µg. (C) Lanes 1 through 18 represent genomic extraction's from (A) following digestion with Sst 1 restriction enzyme. M = 1 Kb DNA ladder purchased from Gibco/BRL®.





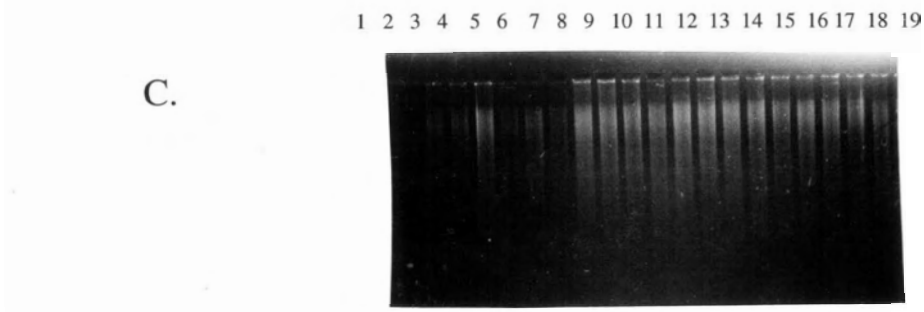


Figure 2: Standard curve derivation for total DNA concentration determination. Known concentrations of Hind III digested Lambda phage DNA (Standard DNA) were measured in duplicate at 260 nm. A standard curve was generated from resulting data. Sample DNA absorbance measurements were performed immediately following generation of standard curve.

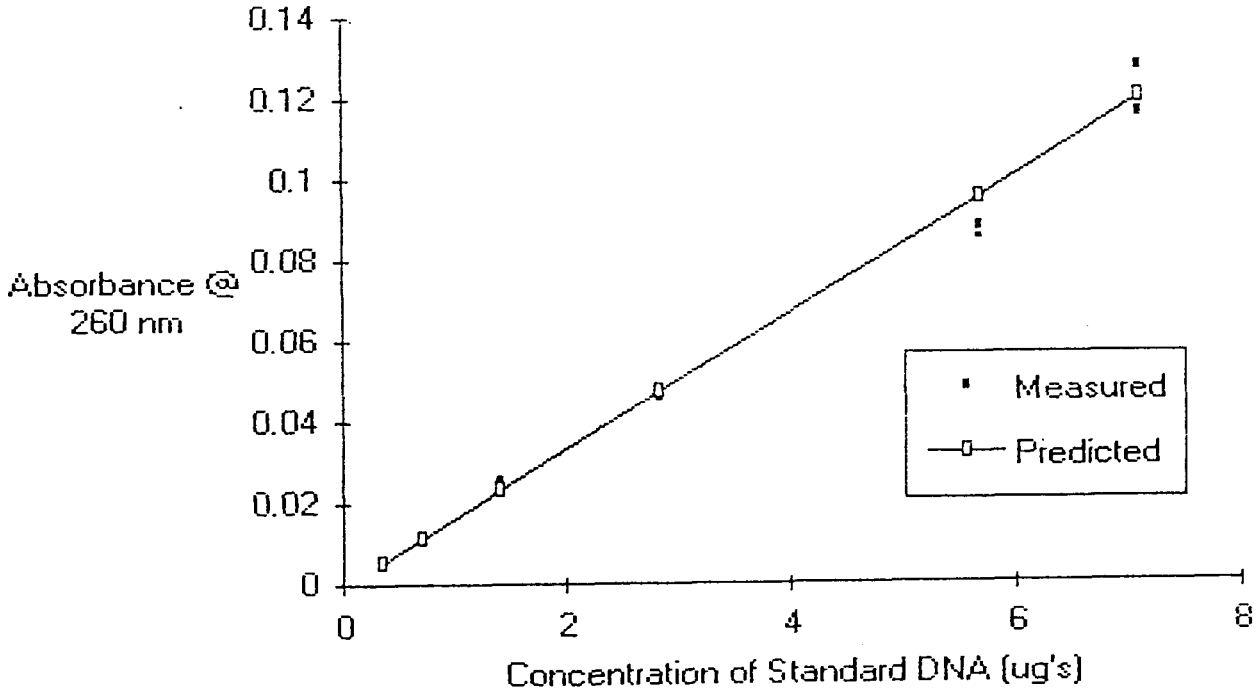


Figure 3: Southern Blot demonstrating low cross reactivity of 958 bp mitochondrial DNA specific probe to non-specific genomic sequences. Lanes 1- 5: control DNA consisting of Sst 1 linearized 16 kb mouse mitochondrial DNA (upper bands) and unlabelled 958 bp mtDNA probe (lower bands) at concentrations of 10, 5, 1, 0.1, and 0.01 ng. Lanes 6 - 9: sample DNA consists of total DNA isolated from 24 month mouse skeletal muscle digested with Sst1 (2, 1, 0.2, and 0.02 μg).

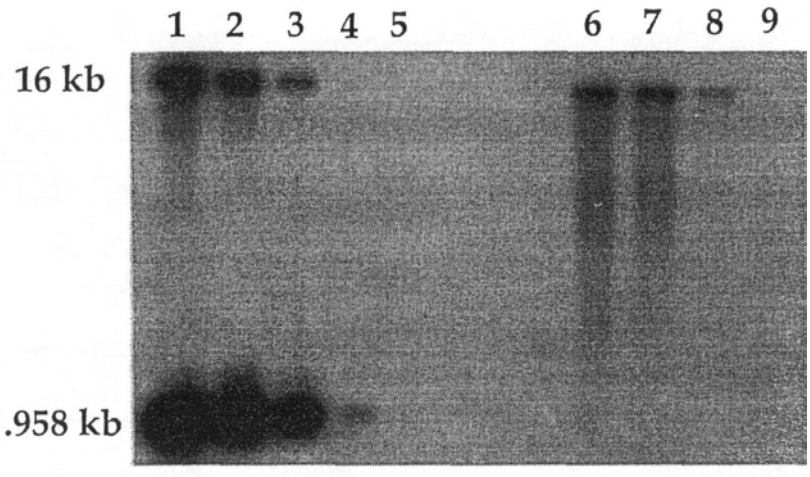


Figure 4: Examples of hybridized slot blots. (A) Rows 1 - 4: Control mitochondrial DNA @ 5, 1, 0.1, and 0.01 ng respectively; Rows 5 - 10: 24 month control mouse cardiac genomic DNA (n = 6) @ 250 ng each slot. (B) Rows 1 - 4: as in (A); Rows 5 - 10: 24 month voluntary exercised mouse cardiac genomic DNA (n = 6) @ 250 ng each slot. All standards/samples are represented in triplicate.

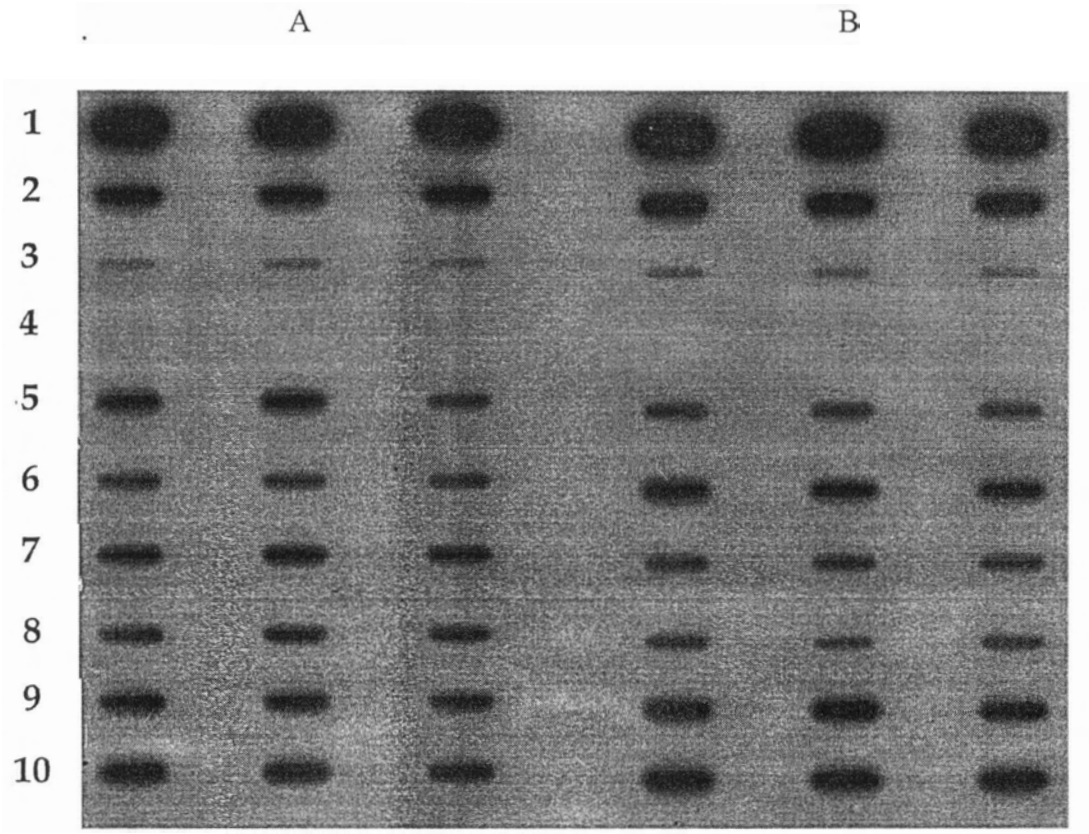
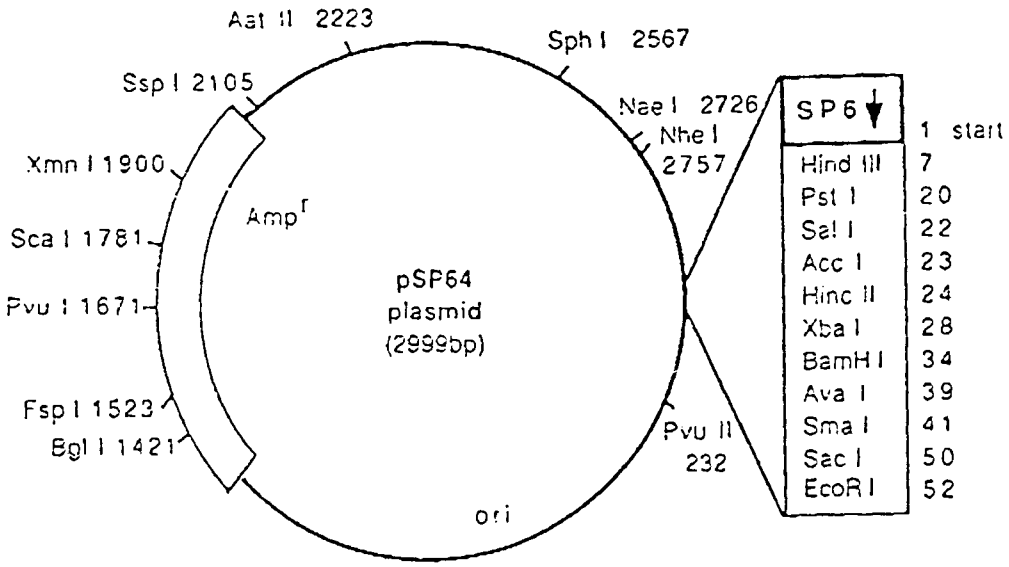
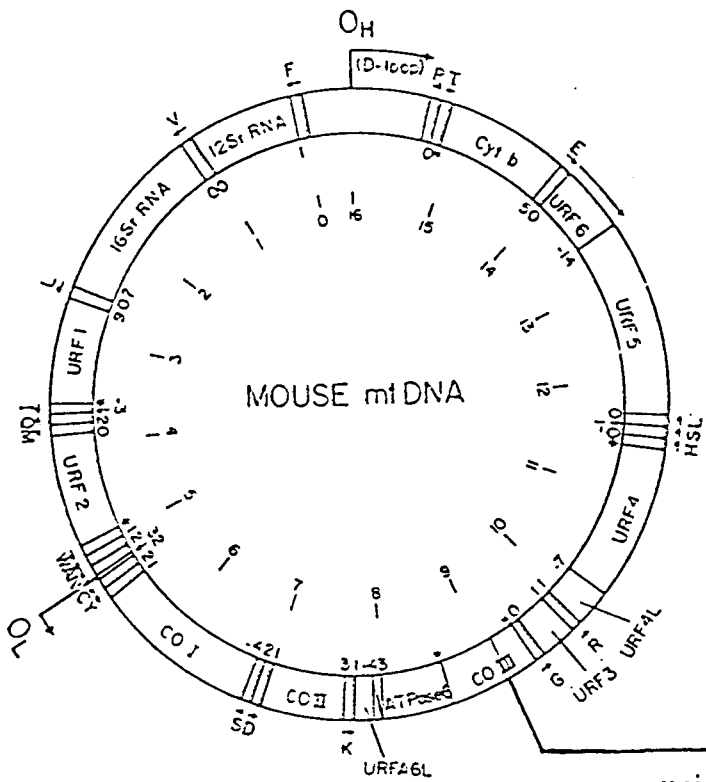


Figure 5: (A) The pSP64 Vector Circle Map (reprinted from Promega® Technical Bulletin). (B) Organization of the Mouse Mitochondrial Genome (reprinted from Bibb *et al*1981). The entire 16295 bp mouse mitochondrial genome was cloned into the pSP64 Sac 1 site by Dr. W. Hauswirth, University of Florida, Gainsville.

A.



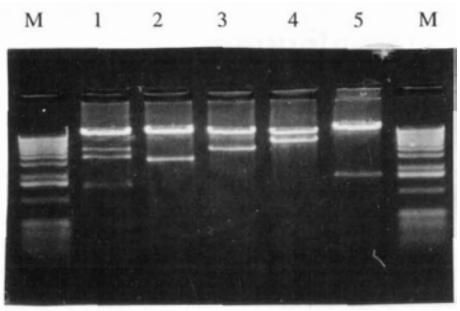
B.



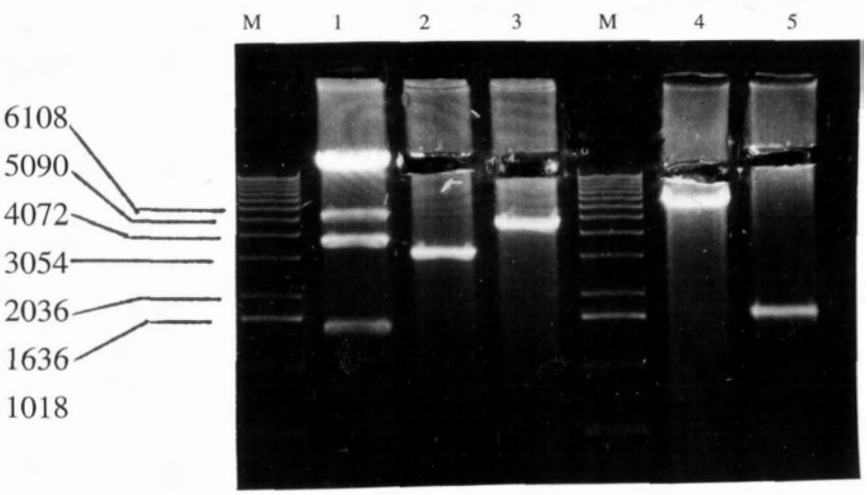
nuc. 9048
unique Sst1 cute site
vector cloning position

Figure 6: (A) Gel depicting various restriction enzyme digests of pSP64/16 kb mouse mitochondrial genome clone. Lane 1: Afl II, incomplete digestion, expected bands @ 14244, 3599, & 1451; lane 2: Bst XI, bands 16055 & 3239; lane 3: Cla I, bands 14445 & 4849; lane 4: EcoRV, bands 12452 & 6842; lane 5: Sac II, bands 17604 & 1690. (B) Lanes 1 through 5 depict identical digests as in (A) with better resolution. Upper bands were cut out for construction of deletion clones. (C) Lane 1: plasmid liberation from Sst I digest of pSP64/16Kb clone; bands 16295 linearized mouse mitochondrial DNA & 2999 bp pSP64 plasmid. M = 1Kb DNA ladder.

A.



B.



C.

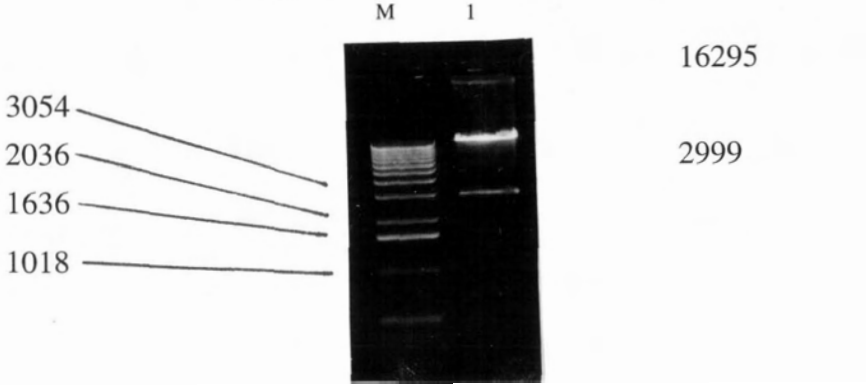


Figure 7: Simplified schematic of the Polymerase Chain Reaction. Pairs of oligonucleotides are specifically constructed complementary to each template DNA strand and orientated to span the DNA to be amplified. Through several cycles (denature, anneal, polymerization) a major amplification product emerges which can be verified through RFLP with restriction endonucleases.

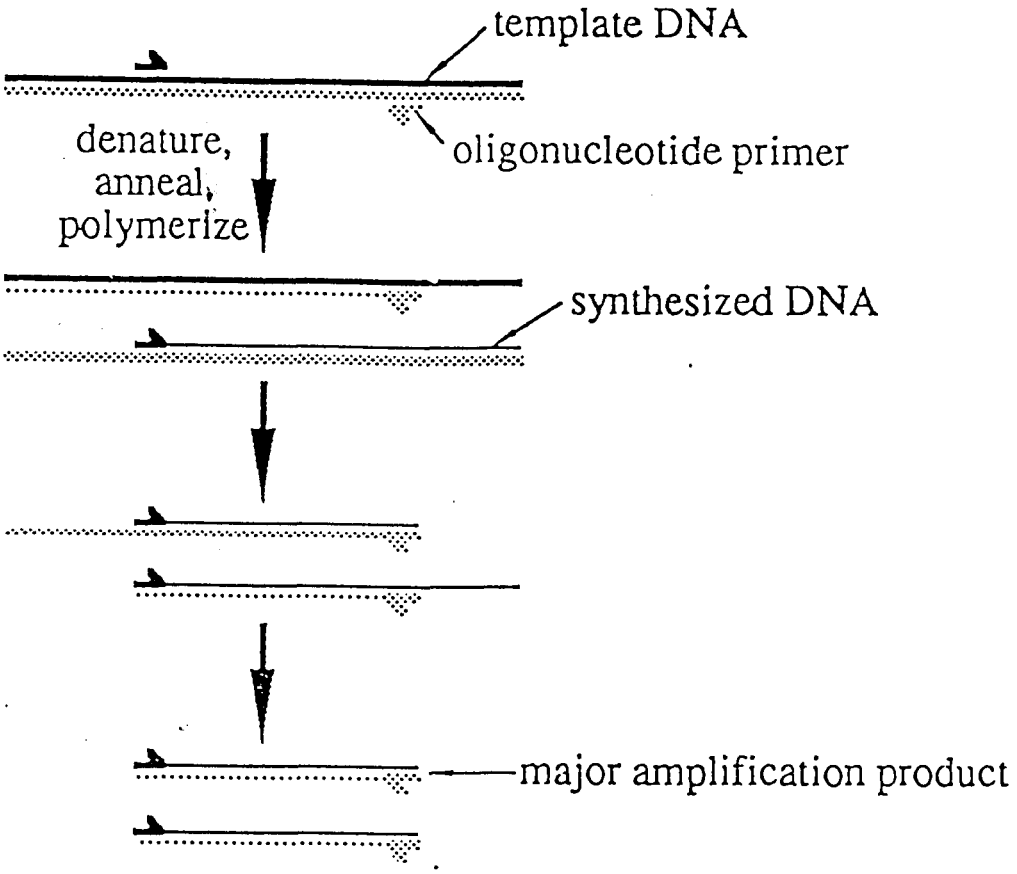
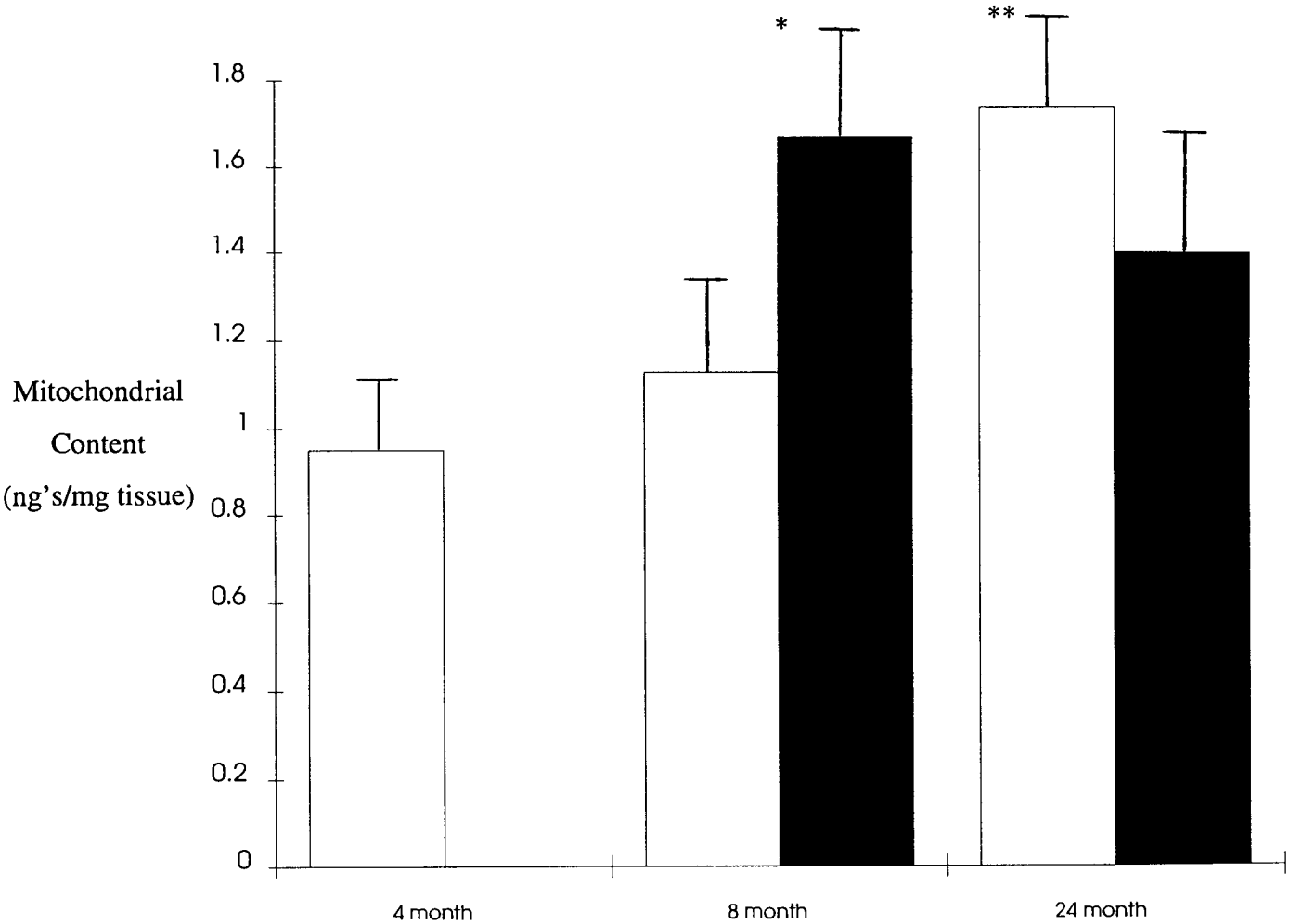


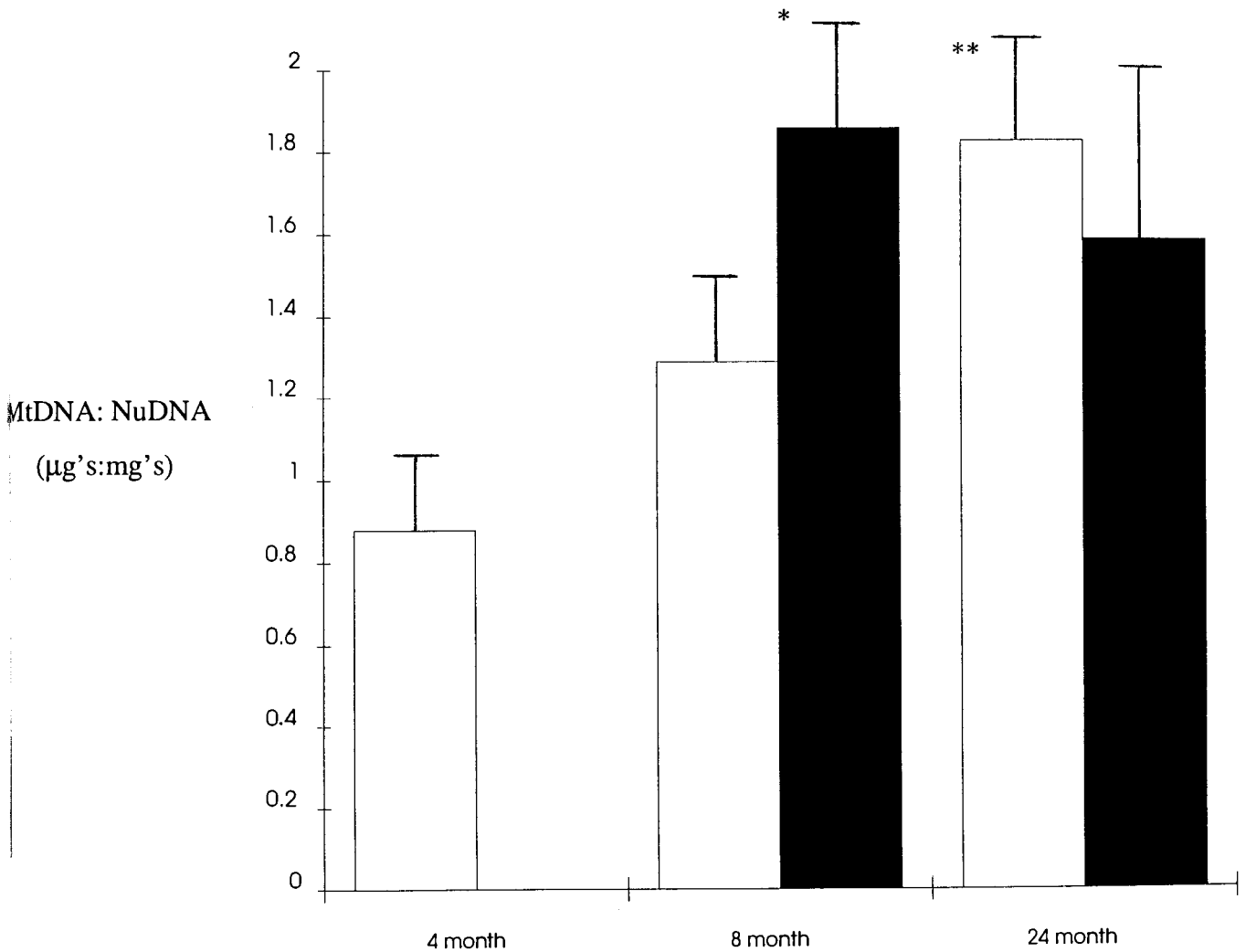
Figure 8: Mitochondrial DNA content changes in mouse cardiac tissue with age and exercise. Clear boxes denote control values; hatched boxes denote voluntary exercise values. Mitochondrial DNA content increases significantly with age.



* Marginal significance from 8 month control values ($P \leq 0.08$).

** Significant increase from 4 month control values ($P \leq 0.05$).

Figure 9: Mitochondrial DNA to nuclear DNA ratio changes in mouse cardiac tissue with age and voluntary exercise. Clear boxes denote control values; hatched boxes denote voluntary exercise values. MtDNA:nucDNA ratio increases significantly with age and with voluntary exercise in 8 month old mice. See table 2 for numerical data.



* Significant increase from 8 month control values ($P \leq 0.05$).

** Signifcant increase from 4 month control values ($P \leq 0.05$).

Figure 10: Primer sequence information for L4984 and H5942 and orientation with respect to the mouse mitochondrial genome. 'L' denotes light strand sequence, complementary to the heavy strand. 'H' denotes heavy strand sequence, complementary to the light strand.

L4984 5'- AAG CCC TAA GAA AAC ACA CA - 3'

H5942 5'- GCC TGC GGC TAG CAC TGG TA - 3'

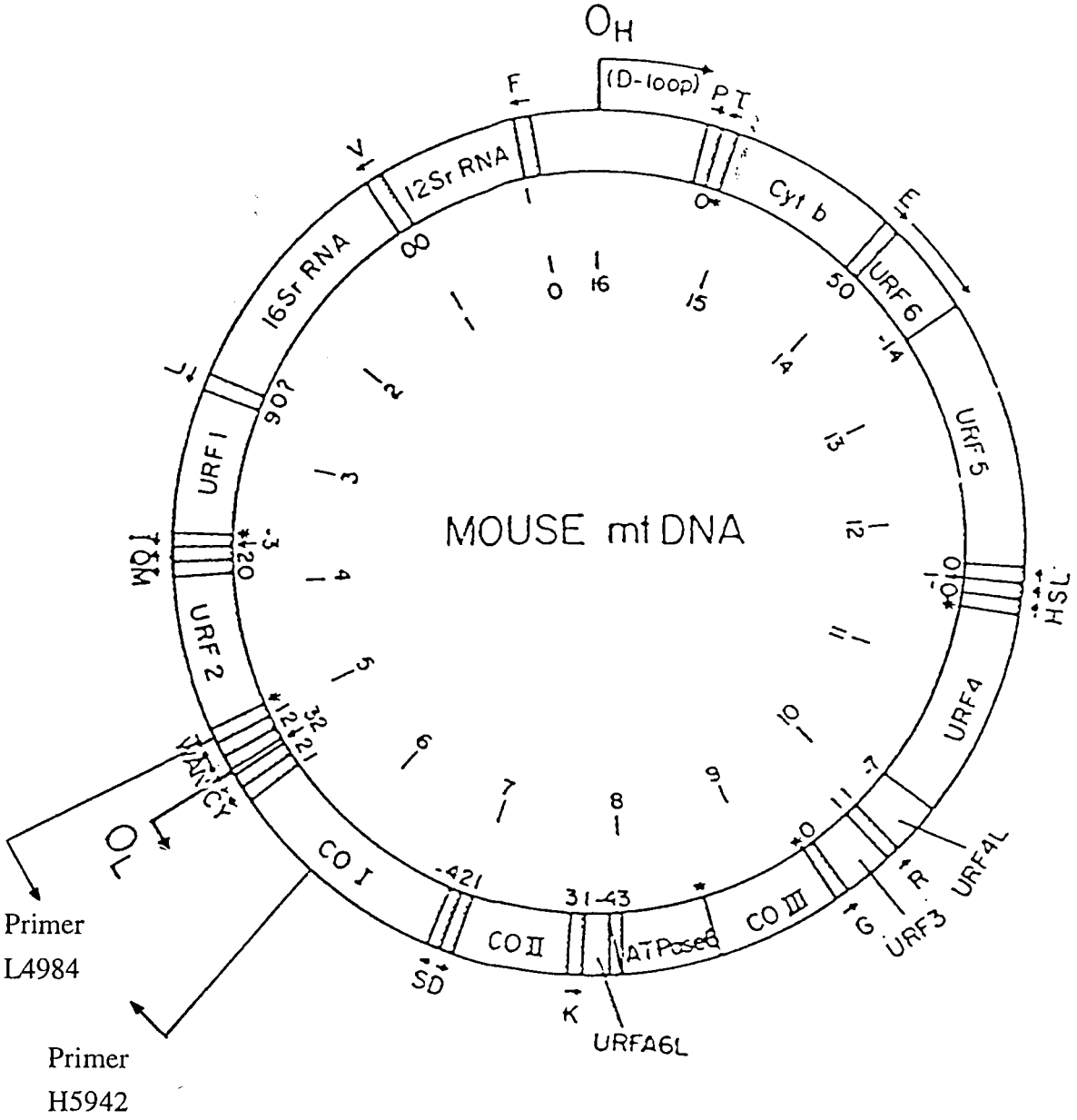


Figure 11: Multiple PCR reactions using primers L4984 and H5942 yeilding a 958 base pair product. Reaction conditions were as described in Methods: Mitochondrial Specific Probe. Mitochondrial DNA template was derived from the indicated sources. Lane 1: Sst I linearized pSP64/mtDNA; Lane 2 & 3: 24 month mouse cardiac total DNA isolation; Lane 4 & 5: 8 month mouse cardiac total DNA isolation; Lane 6: L4984 and H5942 primers only (negative control). M = 1 Kb ladder (BRL©).

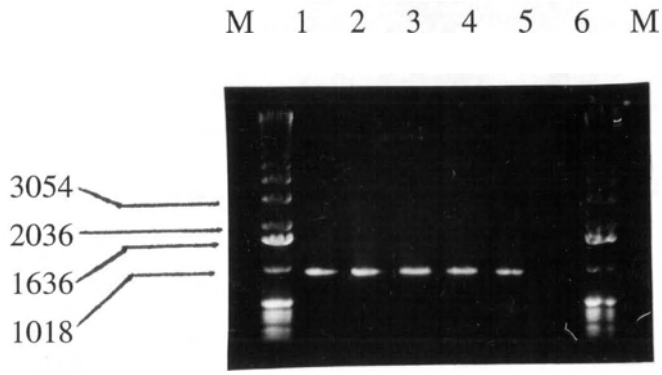
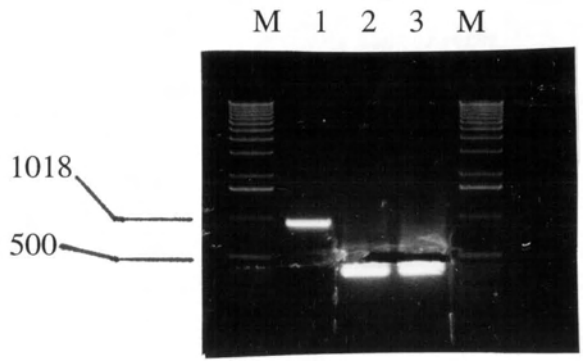
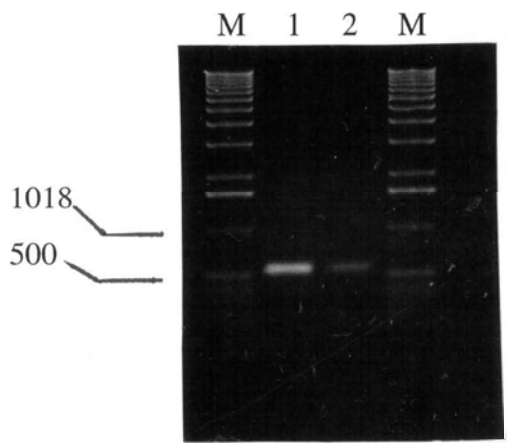


Figure 12: Isolation of 574 base pair CO I sequence from 958 base pair PCR product. A) Lane 1: uncut 958 base pair PCR mitochondrial DNA fragment. Lane 2 & 3: EcoR V digest of 958 base pair PCR fragment; 574 base pair band isolated from gel. B) Lane 1 & 2: Isolated 574 base pair fragment verification.

A.



B.



APPENDIX II

COMPILATION OF TABLES

TABLE 1. Mouse cardiac changes with age and voluntary exercise.

AGE/TREATMENT GROUP	DISTANCE (km/day)	VELOCITY (m/min)	HEART MASS (mg wet tissue)	BODY MASS (grams)	HEART BODY RATIO (X 100)	HEART CS ACTIVITY (μmol/g/min)	SOLEUS MASS (mg)
4 Month Control	N/A	N/A	76.7±3.3	22.5±0.2	0.34±0.02	83.3±6.5	7.0±0.1
8 Month Control	N/A	N/A	[a]108.3±4.8	[a]25.0±1.3	[a]0.44±0.01	78.6±2.3	7.0±0.2
24 Month Control	N/A	N/A	[ab]133.3±4.9	[ab]30.0±0.6	[a]0.45±0.03	75.2±6.5	[a]7.9±0.2
8 Month Voluntary Exercise	1.9±0.3	15.2±0.9	116.7±6.1	25.8 ± 0.6	0.45±0.03	[c]89.3±4.2	[c]8.5±0.2
24 Month Voluntary Exercise	2.1±0.3	8.6±0.4	[c]145.0±4.3	[c]31.0±0.9	0.47±0.02	71.5±2.0	[c]9.4±0.3

All values are reported as mean ± SEM.

(a) Significant increase from 4 month control value (P≤0.05).

(b) Significant increase from 8 month control value (P≤0.05).

(c) Significant increase from age-matched sedentary control value (P≤0.05).

TABLE 2. Mitochondrial content alterations with age and voluntary exercise.

AGE/TREATMENT GROUP	N	Mitochondrial Content (ng mtDNA/mg wet tissue)	MtDNA:NuDNA Ratio (μg : mg)
4 Month Control	6	0.95 ± 0.18	0.88 ± 0.17
8 Month Control	6	1.13 ± 0.22	1.29 ± 0.19
24 Month Control	6	[a]1.74 ± 0.22	[a]1.83 ± 0.25
8 Month Voluntary Exercise	6	[b]1.67 ± 0.26	[c]1.86 ± 0.23
24 Month Voluntary Exercise	6	1.40 ± 0.30	1.59 ± 0.39

All values are reported as mean ± SEM.

(a) Significant increase from 4 month control values (P≤0.05).

(b) Marginal significance from 8 month control values (P≤0.08).

(c) Significant increase from 8 month control values (P≤0.05).