GENOMIC AND BIOCHEMICAL ANALYSIS OF OXIDATIVE STRESS IN BIRDS WITH DIVERSE LONGEVITIES

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ABSTRACT

The relationship among oxidative stress, mitochondrial DNA integrity, and longevity continues to be without a general consensus. Here, we hypothesize that short- and long-lived birds, including the budgerigar (*Melopsittacus undulatus*), guineafowl (*Numida meleagris*), quail (*Corturnix japonica*), and turkey (*Meleagris gallopavo*) differ in oxidative stress measured by blood markers and that this difference correlates with mitochondrial genomic integrity both within and among species. In preliminary studies and to establish a reference and standard for the search for single nucleotide polymorphisms (SNPs), we used a combination of experimental and *in silico* tools for genome analysis to screen selected regions of the chicken (*Gallus gallus*) mitochondrial genome (mtGenome) for SNPs. A total of 113 SNPs was identified which formed 17 haplotypes. The length of the turkey mtGenome sequence developed was 16,967 bp in length, while that of the budgie was 18,193 bp. Annotation of both sequences revealed a total of 13 genes and 24 RNA (22 tRNA and 2 rRNA). Within the budgie mtGenome sequence, a duplicated control region was observed, and there was an additional nucleotide in the NADH dehydrogenase subunit 3 sequence of both the turkey and budgie. The total number of SNPs within the D-loop and 16S rRNA in each of the four species ranged from zero in the quail to 22 in the budgie. The new mtGenome sequences revealed that the turkey was most closely related to

the chicken and quail, and the budgie was closest to kakabo (*Strigops habroptilus*). Oxidative stress, estimated by biomarkers thiobarbiturate acid reacting substance (TBARS), plasma uric acid (PUA), and glutathione (GSH) and at 10, 30, 55, and 80 wks-of-age within each species, was not consistent. The level of GSH was highest in guineafowl, but lowest in budgie. While PUA, an antioxidant, exhibited a significantly ($P<0.05$) decrease as birds grew order, TBARS, a lipid peroxidation index, increased with age. In general, oxidant and antioxidant status appeared to vary among species and to be significantly affected by age, unlike mutations in the mtDNA which remained the same in younger and older birds. This primary findings and discoveries of this dissertation research include the large scale SNP discovery in previously described and novel avian mtGenomes including the chicken and turkey, the two main poultry species, and the determination that oxidative stress in birds appears to vary with age but that this does not affect mitochondrial DNA variation. Recent evidence of work in mice appears to support results described in this dissertation that mitochondrial DNA mutations do not increase with age, the central paradigm of the "Free Radical Theory of Aging". The dissertation also described resources and data that will be a foundation for the use of birds, especially the budgie, as a model for testing this theory that remains of interest to both agricultural and biomedical sciences.

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CHAPTER 1

Introduction

1.1 Oxidative stress

Oxygen is indispensable to the lives of most organisms because ATP production is linked to the reduction of oxygen to water, the primary energy-producing pathway of the cell. During the process of energy metabolism, approximately two percent of the oxygen consumed is converted to free radicals or reactive oxygen species (ROS, Harman, 1983). Typical ROS species include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH, Finkel and Holbrook, 2000). Reactive oxygen species are derived from superoxide leakage from the electron transport chain (ETC), and other cellular activities including the autooxidation of various small molecules (e.g. flavins, catecholamines, and hydroquinones), the microsomal cytochromes P450, and microsomal flavoprotein reductases (Halliwell and Gutteridge, 1989; Kleinveld et al., 1989). Superoxide and hydrogen peroxide are relatively unreactive and long-lived in biological systems but their danger lies in the fact that they readily give rise to highly reactive hydroxyl radicals (·OH) which are involved in numerous forms of damage to cellular macromolecules (Kleinveld et al., 1989). These free radicals cause cell damage to macromolecules including DNA, proteins, and lipids. They also alter metabolism as much as actual cell damage by diminishing the supply of NADPH available for important physiological processes (Rodriguez and Redman, 2005).

Animals have developed a strategy to counter free radicals by producing enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) which remove superoxide and peroxides before they react with other catalysts to form more reactive species. Non-enzymatic antioxidants include water-soluble ascorbate, glutathione, uric acid, and lipid-soluble vitamin E, ubiquinone, and β-carotene. Non-enzymatic antioxidants function by terminating the peroxidative chain reactions initiated by ROS released from enzymatic degradation (Miller et al., 1993). Despite the collective effort of all these antioxidants, the defenses they provide are not completely effective as levels of ROS can be detected under normal physiological conditions (Chance et al., 1979). Moreover, the efficiency of these protective systems have been hypothesized to decrease with age which indicates that both free radical generation and declining antioxidant defense have to be considered as potentially important contributors to the aging process (Wickens, 2001).

A sophisticated antioxidant defense system counteracts and regulates overall ROS levels to maintain physiological homeostasis (Finkel and Holbrook, 2000). A decrease in ROS levels below a homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defense. For example, decreased ROS generation by phagocytic cells can result in a defect essential host defense mechanism which is necessary to combat infection (Finkel and Holbrook, 2000). Similarly, increased ROS in animals is correlated with two potentially important effects: damage to various cell components and triggering of the activation

of specific signaling pathways. Both of them can influence numerous cellular processes linked to aging and the development of age-related diseases.

Organisms that experience wide variation in oxygen availability deal with the accompanying wide variation in the production of ROS by two major processes. The first would be to maintain constitutively high levels of antioxidant defenses (enzymes, low molecular weight antioxidants) so that any stress can be dealt with effectively. The second would be to elevate antioxidant defenses as a rapid response to anoxia/ischemia stress so that there are enough antioxidant anticipation of the overgeneration of ROS during the aerobic recovery period (Storey, 1996a). Studies have suggested that both are used in different systems with the "choice" determined by the extent to which a species experiences oxidative stress naturally. For example, a good facultative anaerobe usually deals with the inevitable burst of ROS generation during the anoxic to aerobic transition by maintaining high activities of antioxidant enzymes and a large glutathione pool constitutively (Storey, 1996b). However, animals that only infrequently experience oxidative stress appear to initiate adaptive changes in their antioxidant defenses during the stress period that minimize oxidative damage during recovery (Lutz and Storey, 1996).

1.2 Biomarkers for oxidative stress

There are multiple, complex methods for assessing free radical activities. The choice of proper biomarkers depends on experimental conditions, the availability of analytical facilities, and the investigator's interest. The major approaches to assess free radical activities can be divided into three catalogs: determination of endogenous antioxidant levels; measurement of the products of oxidized macromolecules; and direct detection of free radicals by electron spin resonance analysis (Jackson, 1999).

For assessing endogenous antioxidant capacity, both non-enzymatic and enzymatic antioxidants are used to assess the oxidative stress. For non-enzymatic, studies have examined the concentrations of antioxidants, such as vitamin E, vitamin C, and glutathione (GSH) in plasma and cells (Griffiths et al., 2002). On the other hand, the cellular activities of antioxidant enzymes including glutathione reductase, SOD, CAT, and GPx are also widely used in most studies (Fang et al., 2002).

The measurement of the products of oxidized macromolecules can be divided in to lipid, protein, and DNA peroxidation (Griffiths et al., 2002). Assessments of lipid peroxidation include the analysis of lipid peroxides, isoprostanes, diene conjugates, and breakdown products of lipids such as malonaldehyde, ethane, pentane, and thiobarbituric acid reactive substances (TBARS, Abuja and Albertini, 2001). F_2-isoprostanes is often used as a reliable marker of lipid peroxidation (Montuschi et al., 2004). For assessing ROS-induced protein oxidation, most investigators have determined the production of protein carbonyls, the loss of free thiol groups in

proteins, and nitration of protein-bound tyrosine residues (Fang et al., 2002). Protein nitrotyrosine has also been widely used as a convenient stable marker for the production of reactive nitrogen-centered oxidants such as NO and peroxynitrite (Gilbert, 2000). Specific products of DNA base oxidation such as 8-hydroxydeoxyguanosine (8-OHdG), 5-OH cytosine, 8-OH adenine, 8-OH guanine, and thymine glycol have been measured often to assess DNA base oxidation (Jackson, 1999). Importantly, urinary excretion of 8-OHdG may provide a useful, non-invasive means to assess whole-body DNA base oxidation in humans and animals (Jackson, 1999).

Direct detection of free radicals has been performed using electron spin resonance and spin trapping techniques (Fang, 1991). Though the electron spin resonance technique is suitable for detecting free radicals in solution chemistry, it has limited application to biological tissues because of their usually high content of water (Griffiths et al., 2002). However, this problem can be overcome by the use of the spin trapping technique, which involves the conversion of highly reactive free radicals to relatively inert radicals, followed by electron spin resonance analysis (Jackson, 1999).

Here in this study, the antioxidant (GSH and uric acid) and oxidized macromolecule (TBARS) were used as biomarkers to assess the oxidative status in birds. Glutathione, the most abundant thiol-containing substance of low molecular weight in cells, is synthesized from glutamate, cysteine, and glycine. *N*-acetylcysteine is a stable, effective precursor of cysteine for intracellular GSH synthesis (Sies, 1999). The hydrolysis of glutamine to glutamate by

glutaminase and the production of glutamate from α-ketoglutarate and branched-chain amino acids via transamination are two major sources of plasma and cellular glutamate for GSH synthesis (Fang et al., 2002). Glutathione can scavenge free radicals with the formation of oxidized glutathione (GSSG), which is converted back to GSH by the NADPH-dependent glutathione reductase (Sies, 1999). Because GSH is rapidly oxidized to GS-SG by radicals and other reactive species and GS-SG is exported from cells, intracellular $[GSSG]:[GSH]^2$ ratio can provide a valid index of oxidative stress (Griffiths et al., 2002). For example, Erden-Inal used this index to assess the aging effect of GSH in human. The results showed that the GSH level had a negative correlation with age, which indicated an increased oxidative stress level.

Uric acid, in addition to being a waste product, is considered a biomarker for many physiological characteristics in vertebrates (Ames et al., 1981). Especially in birds, it cannot be broken down *in vivo* (Simoyi and Klandorf, 2003). It is considered an important antioxidant (Johnson et al., 2001) as plasma uric acid (PUA) has been shown to scavenge free radicals (Whiteman and Halliwell, 1996), to chelate transition metals (Rowley and Halliwell, 1985), and to block peroxynitrite, a toxic product of free radical reaction with nitric oxide (Whiteman and Halliwell, 1996). As an antioxidant, PUA is considered to affect oxidative stress (Cutler, 1984). Hartman et al. (2006) reported differences among turkey varieties in PUA. The results suggested that differences in basal levels of PUA in physiologically normal birds were influenced by sex, weight, and genetic background but may be independent of age.

The sensitivity of measuring TBARS has made this method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress (Lef'evre G. et.al, 1998). It has been modified by researchers for use with many types of samples including drugs, food products and human and animal biological tissues (Janero, 1998; Callaway et al., 1998; Jentzsh et al., 1996; Jo et al., 1998). The level of TBARS has provided important information regarding free radical activity in disease states and has been used for measurement of antioxidant activity of several compounds (Villa-Caballero et al., 2000). Although much controversy has appeared in the literature regarding the specificity of TBARS toward compounds other than MDA, it remains the most widely employed assay used to determine lipid peroxidation (Armstrong and Browne, 1994).

1.3 Theories of aging

Aging is the post-maturational processes that lead to diminished homeostasis and increased organismic vulnerability (Toren, 2003). The major characteristics include: increased mortality with age after maturation (Kaeberlein et al., 2001), changes in biochemical composition in tissues with age (Shock et al., 1984; Riggs and Melton, 1986), progressive decrease in physiological capacity with age (Shock, 1985), reduced ability to respond adaptively to environmental stimuli with age (Adelman et al., 1978), and increased susceptibility and vulnerability to disease (Brody and Brock, 1985).

Evolutionary pressures have selected for successful reproduction, making it likely that the post-reproductive physiology of an organism, such as aging, is an epigenetic and pleiotropic manifestation of the optimization for early fitness. Indeed, antagonistic pleiotropy, where genes that enhance early survival and function but are disadvantageous later in life, may play an overriding role in aging. No one unifying theory may exist, since the mechanisms of aging could be quite distinct in different organisms, tissues, and cells. The theories of aging can be divided into development-genetic theories and stochastic theories (Toren, 2003).

The development-genetic theories consider the process of aging to be part of the genetically programmed and controlled continuum of development and maturation, which include longevity genes, accelerated aging syndromes, neuroendocrine theory, immunologic theory, cellular senescence, and cell death. For example, the theory of longevity genes refers to that the longevity is under genetic control (Finch and Tanzi, 1997). Though the degree of heritability is likely to be less than 35%, studies have revealed that genetic mutations can significantly modify senescence in yeast (Jazwinski, 1996), *Drosophila melanogaster* (Lin et al., 1998) and mammals (Jazwinski, 1996). The neuroendocrine theory proposes that functional decrements in neurons and their associated hormones are central to the aging process (Mobbs, 1996). At the meantime, the theory of cellular senescence hypothesize that the aging is a cellular as well as an organismic phenomenon, and that the loss of functional capacity of the individual reflected the summation of the loss of critical functional capacities of individual cells (Hayflick and Moorhead, 1965).

The stochastic theories propose that aging is caused by random damage, such as oxidative stress, to vital molecules. The damage eventually accumulates to a level sufficient to result in the physiological decline associated with aging (Toren, 2003). These theories include somatic mutation and DNA repair, error-catastrophe, protein modification and free radical theory of aging. The DNA repair theory hypothesizes that the ability to repair DNA damage in cell cultures derived from species with a variety of different lifespans correlates directly with the longevities (Hart and Setlow, 1974). The error-catastrophe theory proposes that random errors in synthesis eventually occur in proteins, DNA or other "template" molecules. This could result in an amplification so that the subsequent rapid accumulation of error-containing molecules would result in an "error-catastrophe" that would be incompatible with normal function and life (Orgel, 1963). Kohn (1978) and Bjorksten (1974) hypothesized that the accumulation of post-translationally altered proteins could impair cellular, and ultimately, organ function, and this is the protein modification theory.

All of the stochastic theories can be associated with free radical theory of aging (Harman, 1956, 1981), because most often, it is the free radicals that cause damage to biomolecules. This theory pointed out that: aging and the age-related diseases associated are basically due to the deleterious side attacks of free radicals on cell constituents and on the connective tissues. The free radicals arise largely through oxygen consumptions in the cell by the oxidative enzymes and in the connective tissues by traces of metals such as iron, cobalt, and manganese. There are several other theories divided from free radical theory of aging, including oxidative stress

hypothesis of aging and redox mechanism of mitochondrial aging or mitochondrial DNA/ oxidative stress hypothesis.

1.4 Mitochondria, oxidative stress, and aging

Mitochondrion, a sub compartment of the cell, is bound by a double membrane and has several components that allow it to function as the primary site for oxidative phosphorylation in eukaryotes (DiMauro, 2004). It has a genome (mtGenome) independent of the nucleus and is approximately 17 kb in size. The mtGenome encodes 22 tRNAs, 2 rRNAs, and 13 polypeptides, all of which function primarily in ETC, (Chinnery and Schon, 2003). Because the mtGenome is maternally inherited, the DNA sequence is also used extensively as a phylogenetic tool to infer relatedness as well as evolutionary lineages. Its primary role, however, remains as the power house in eukaryotic cells.

Mitochondria link the energy-releasing activities of electron transport and proton pumping with the energy conserving process of oxidative phosphorylation, to harness the value of foods in the form of ATP. It has been estimated that more than 90% of cellular oxygen is consumed in mitochondria for energy metabolism, and 2% of the consumed oxygen is converted into ROS. At levels above certain thresholds, ROS can detrimentally alter mitochondrial and non-mitochondrial macromolecules including lipids, proteins, and DNA (Lee et al., 1997). The ROS damage to DNA is considered to be the highest because of the close spatial relationship

between mitochondrial DNA (mtDNA) and the free radical-generating system located in the inner mitochondrial membrane (Miquel et al., 1980; Sastre et al., 2003).

Mitochondrial DNA is maternally transmitted, continues to replicate throughout the lifespan of an organism in both proliferating and post-mitotic (non-proliferating) cells, and is subject to a much higher mutation rate than nuclear DNA. This is due, in large part, to inefficient repair mechanisms: unlike nuclear DNA, the mtGenome lacks histones which offer protection against free radicals. Damage to mtDNA caused by ROS or oxidative stress includes deletions and point mutations, which have been shown to affect aging and age-related diseases in vertebrates (Linnane et al., 1989). Examples of diseases associated with damage to the mtDNA include Alzheimer's, Parkinson's (Troen, 2003), diabetes (Maassen et al., 2004), and skeletal and cardiac myopathies (Zeviani and Donato, 2004).

The mtDNA/oxidative stress hypothesis represents the relationship between oxidative stress, mtDNA mutation and aging, and it is a synthesis of several theories and therefore comprises elements of both stochastic and developmental-genetic mechanisms of aging. It is proposed that reactive oxygen species contribute significantly to the somatic accumulation of mtDNA mutations, leading to the gradual loss of bioenergetic capacity and eventually resulting in aging and cell death (Linnane et al., 1992; Fleming et al., 1982; Wallace, 1992).

While this relationship still remains a theory, several studies have provided experimental evidence for the link between mtGenome integrity and aging. For example, Barja and Herrero (2000) evaluated mtDNA damage and longevity of different animals. Using 8-oxodG as a

biomarker for oxidative DNA damage, they compared short-lived species including the mouse, which lives only 3.5 years, to long-lived species, including the horse, with an average life span of 46 years. The comparisons revealed that there was no significant link between the level of 8-oxodG/10^5dG in nuclear DNA and maximum life span (MLSP). However, there was an inverse correlation between 8-oxodG/10^5dG in mtDNA and MLSP. A similar relationship between life span and mtGenome integrity was described in an earlier comparative study by Herrero and Barja (1999). Again, using 8-oxodG level as biomarker for mtDNA damage, long-lived birds of similar body size and metabolic rate as short-lived mammals had lower oxidative damage.

Despite these associations proposed by mtDNA/oxidative stress hypothesis, however, the mechanisms by which mtDNA mutations influence aging and age-related diseases remain little understood. Some studies suggest that impaired respiratory function, which affects ATP synthesis and further increases the production of free radicals, is a possible mechanism (Wallace, 2000). The impaired respiratory function caused by the increased ROS production may also cause dysfunction of the ETC enzyme complexes (Linnane et al., 1998). For example, Moreno-Loshuertos et al. (2006) showed that the differences in ETC enzyme complex performance was masked by a specific upregulation in mitochondrial biogenesis, triggered by an increase in generation of ROS in mice cells carrying a SNP generating ten adenines on the D-loop of tRNA arginine in mtGenome.

Recent evidence, however, suggests that the relationship between mtGenome integrity and aging may be independent of ROS production (Kujoth et al., 2005). Results of the study

showed that deletions in mtDNA did indeed accelerate aging, but contrary to the free radical theory of aging, the effect was mediated through apoptosis but not ROS. Another more recent study (Vermuslt et al., 2007) showed that by measuring the *in vivo* rate of change of the mtGenome at a single-base pair level in mice, the mutation frequency in mouse mitochondria was more than ten times lower than previously reported. Although there was an 11-fold increase in mitochondrial point mutations with age, a mitochondrial mutator mouse was able to sustain a 500-fold higher mutation burden than normal mice, without any obvious features of rapidly accelerated aging. Thus, their results strongly indicated that mitochondrial mutations did not limit the lifespan of wild-type mice.

1.5 Birds, oxidative stress, and longevity

The vertebrate animal models currently best developed for studies of the relationship between oxidative stress and aging including age-related diseases are laboratory rats and mice. They are highly suitable for use in controlled experiments, but, ironically, are poorly adapted for aging. Tolerance to oxidative stress in short-lived species such as mice and rats could conceivably differ qualitatively, as well as quantitatively, from those in much longer-lived organisms (Holmes, 2004).

Domestic and cage birds are already used widely in biomedical research, and they are inexpensive to maintain. Birds, as mammals, are warm-blooded animals, any anti-aging

mechanisms they possess could be similar to specific molecular mechanisms of aging in mammals. Moreover, since the longevities of some birds approach those of humans, avian aging processes might actually be more similar to ours in some respects than those of the short-lived rodents typically used to model basic mammalian aging processes (Holmes and Ottinger, 2003). Domestic birds from several different orders are economical and feasible to maintain and breed. Since birds are egg-layers, embryonic cells and tissues are readily harvested for *in vitro* studies. Birds are also prone to many of the same diseases of aging afflicting mammals, including infertility, cardiovascular disease, cancers, cataracts, osteoarthritis, and diabetes (Austad, 1997).

The best candidate species as avian laboratory models in most cases are small, domesticated birds in several different orders, for which husbandry practices are well established and maintenance costs nearly as economical as for laboratory rodents (Austad, 1997). They include short-lived species, like the Japanese quail (average life span 3-4 years), and several popular pet species, like the budgerigar (a small parrot), and the canary (a finch), both of which can live up to 20 years or so. Lifetime energy expenditures for all these birds exceed those of comparably-sized rodents (Holmes and Ottinger, 2003). For example, the Ottinger laboratory has used the Japanese quail extensively as a model for aging-related declines in reproduction, focusing on a variety of changes in behavioral, gonadal, metabolic, sensory, and central neuroendocrine function (Ottinger, 1991, 2001).

Birds tend to live longer than would be predicted by their metabolic rates (Jacobs, 2003). The maximum life spans of birds are generally 2-3 times those of similar-sized mammals

(Holmes, 2004). Literature reports suggested that the lifespan of birds ranges from 0.2 to 73 for plain chachalaca and African grey parrot, respectively (Flower, 1938; Clapp et al., 1982). Their high lifetime energy expenditures (up to 8 times those of mammals of similar size) suggest birds have unusually effective adaptations for preventing oxidative (Holmes, 2004). In the course of their longer lives, therefore, birds process much more oxygen per cell than do mammals, including long-lived mammals such as humans (Ogburn et al., 2001). There is growing evidence that bird cells in culture are exceptionally resistant to oxidative damage (Holmes, 2004). Two reasonable mechanisms could explain the longevity difference between birds and mammals, assuming that oxidative damage is a major determinant of longevity. First, birds might produce fewer ROS per mole of oxygen consumed. Second, long-lived birds possess superior defenses of an unknown nature against oxidative damage, which might correlate with the integrity of mtGenome (Ogburn et al., 2001). It is essential to explore the mechanisms underlie the little understood avian model, and it can give important resource for mammalian oxidative stress-related aging studies.

1.6 Dissertation research hypothesis and objectives

Based on the "free radical theory of aging" and "mtDNA/oxidative stress hypothesis", here, we hypothesized that oxidative stress was the primary biological mechanism responsible for differences in longevity among birds and that this difference correlated with mtGenome

integrity both within and among species. To test this hypothesis, my dissertation has three specific aims: determine if birds of varying longevities have different levels of oxidants and antioxidants that are biomarkers for oxidative stress; compare mitochondria genomic DNA sequences of birds of varying longevity to identify mutations or polymorphisms; carry out an association studies between oxidative stress and mtDNA mutations.

To achieve these aims, the preliminary study was first carried out for two considerations: the source of mtDNA and a reliable method for amplifying and screening mtDNA for mutations. In the first chapter of this dissertation, model species chicken (*Gallus gallus*) was used to establish a standard and reference for the mtGenome mutation analysis. I used this experiment method together with *in silico* tools to identify nucleotide variants in the chicken mtGenome including the coding and non-coding (D-loop) regions. The distribution of the experimentally identified mitochondrial DNA variants in broilers and White Leghorn chickens was also assessed.

The avian species that we chose include budgie (*Melopsittacus undulatus*), guineafowl (*Numida meleagris*), quail (*Corturnix japonica*), and turkey (*Meleagris gallopavo*). Since there is no complete mtGenome sequences of two of these four species, budgie and turkey, the mtGenome sequences of them were determined to facilitate the SNP analysis, which were described in the second and third chapters. These sequences were also used to carry out phylogenetic studies which would help the validation of the genome and add biological value to the information.

To determine if one or both of oxidative stress and mtGenome mutation underlie differences in longevity observed in birds, I used both oxidant and antioxidant biomarkers to assess the oxidative status. I evaluated the levels of GSH, PUA and TBARS as indicators of specific oxidative stress status in avian species with varying longevities at different ages. I also detected the SNPs in specific fragments on 16S rRNA and D-loop in mtGenome in these avian species to find out the association among mtGenome integrity, oxidative stress and aging. All of these were reported in the fifth chapter of this dissertation.

My research provided evidence that may support some recent studies that point mutations in the mtGenome may not be associated with the severity of oxidative stress in birds, but oxidative stress was significantly correlated with age though not consistent. The data generated for oxidative stress, established basal levels of biomarkers in normal birds which can be used as references in diseases such as cardiomyopathy. The polymorphisms detected in birds could also be important resources for the genotype:phenotype studies.

CHAPTER 2

Mitochondrial DNA Sequence and Haplotype Variation Analysis in the Chicken, *Gallus gallus*

2.1 Abstract

Though it could be useful for genotype:phenotype assignment, our knowledge of the nature and extent of variation in the chicken (*Gallus gallus*) mitochondrial genome (mtGenome) is limited. In this preliminary study to establish a reference and standard for the search for single nucleotide polymorphisms (SNPs), we used experimental and *in silico* tools to identify nucleotide variants in the mtGenome including the coding and non-coding (D-loop) regions. The distribution of the experimentally identified mitochondrial DNA variants in broilers and White Leghorn chickens was also assessed. A total of 113 SNPs was identified. The *in silico* analysis revealed a total of 91 SNPs, with 70 in the coding region, and 21 in the non-coding region. Of the 41 experimentally identified SNPs, 27 were in the D-loop. Nineteen of the 91 SNPs detected in the *in silico* analysis were also observed in the experimental SNP analysis. Together, the experimentally identified SNPs in the non-coding region formed 11 haplotypes while the 14 SNPs in the coding region formed 6. While nine of the D-loop region haplotypes were not observed in the White Leghorn chickens, three of the six haplotypes from the coding region occurred at a significantly higher frequency in broilers. To our knowledge, this investigation represents the first whole mtGenome scan for variation and an evaluation, though limited in

sample size, of the haplotype distribution in meat- and egg-type populations using the SNPs and haplotypes identified.

Key word: *Gallus gallus*, mitochondrial genome, single nucleotide polymorphism, haplotypes

2.2 Introduction

Mitochondria are the primary sites for oxidative phosphorylation in eukaryotes (DiMauro, 2004). The mitochondrion has a genome (mtGenome) that is approximately 16-17 kb in size in most animals including birds. The mtGenome encodes 22 tRNAs, 2 rRNAs, and 13 polypeptides, all of which function primarily in the electron transport chain (Chinnery and Schon, 2003). Expression of these genes is essential in vertebrates for energy production, metabolism, cellular homeostasis, and apoptosis (DiMauro, 2004). The importance of the mitochondria in diverse physiological processes makes mutations in the mitochondrial DNA (mtDNA) an important factor in the incidence and severity of diverse diseases and abnormalities in vertebrates (Linnane et al., 1989; Troen, 2003). For example, mutations in mtDNA have been shown to be associated with diseases like Alzheimer's and Parkinson's (Troen, 2003), diabetes (Maassen et al., 2004), non-hereditary tumors (Zanssen and Schon, 2005), and skeletal and cardiac myopathies (Zeviani and Donato, 2004). Another example involves a common C-T variant at nucleotide position 16,189 of human mtGenome that was reported by Khogali et al. (2001) to be associated with susceptibility to dilated cardiomyopathy.

In the chicken, Froman and Kirby (2005) and Froman and Feltmann (2005) described results that appear to correlate sperm mobility with mitochondrial function. A recent review by Wallace (2005) describes mitochondrial mutations with significant effect on vertebrate health. The mutations are categorized as rearrangements, missense, or tRNA and rRNA nucleotide changes (Wallace et al., 1988a, 1988b; Holt et al., 1990). In animals, variation in selected regions

of the mtGenome has been used extensively for phylogenetic studies. For example, Cozzi et al. (2004) used variation in the non-coding region or D-loop to evaluate the genetic relatedness among seven horse breeds. The analysis revealed ancient maternal lineages and high diversity in the mtDNA sequences. In dogs, Savolainen et al. (2002) used D-loop polymorphisms to determine that almost all domestic breeds have a common East Asian origin that can be traced to a single gene pool. Similarly, Pires et al. (2006) used cytochrome *b* and D-loop sequence polymorphism to evaluate the diversity and relatedness among Portuguese native dog breed. The results showed that Iberian and North African dog breeds possessed breed-specific mtDNA haplotypes, and there was no evidence for introgression of North African haplotypes in Iberian dogs.

As in other livestock and poultry, in the chicken, mtGenome variation analysis has been used primarily in phylogenetic studies. For example, polymorphisms in 12S rRNA and cytochrome oxidase subunit I genes were previously used to study phylogenetic relatedness among human, house mouse, and chicken (Mindell et al., 1991). This study, as expected, revealed that the difference between the two mammals was significantly smaller than the differences between the mammals and chicken. Shen et al. (2002) also used cytochrome *b* gene polymorphisms and haplotypes developed from the variants to show that genetic differences within and between chunky broilers and egg-type chickens were significant. Similarly, Niu et al. (2002) used variation in the D-loop region to evaluate the origins and relatedness among native

Chinese chicken breeds. Another study by Komiyama et al. (2004) used D-loop sequence polymorphisms to show that three long-crowing chicken strains share a common origin.

Though limited, some studies have reported the non-phylogenetic use of variation in the livestock and poultry mtGenome. For instance, Li et al. (1998) described a SNP in the NADH dehydrogenase subunit IV gene that appeared to be associated with resistance to Marek's diseases in White Leghorn chickens. A mixed-model analysis in cattle by Mannen et al. (2003) showed a significant association between SNPs in the mtGenome with carcass traits. Central to these studies involving both phylogenetic and association analysis was the SNP discovery in the mtGenome.

Here, we used *in silico* and PCR-based resequencing of mtGenome to screen for DNA polymorphisms in the chicken. Further, the nucleotide variations were used to define novel haplotypes whose relative distributions in commercial egg- and meat-type chickens were also evaluated. This work describes SNPs and haplotypes that could be useful for investigations to define the molecular basis of many metabolic disorders, diseases, and abnormalities that affect chickens.

2.3 Materials and methods

Animals and DNA extraction

A total of 53 birds, including 33 broilers and 20 White Leghorn chickens, was used in the experimental analysis. Pulp was obtained from secondary feathers collected from each bird and used to isolate genomic DNA using a minor modification of the recommended protocol for the DNeasy Tissue Kit (Qiagen, Valencia, CA). The modification included the addition of 100 mg/ml dithiothreitol to facilitate lysis of the feather pulp during incubation.

SNP analysis

A PCR-based resequencing method (Smith et al., 2001) was used to scan the chicken mtGenome for SNPs. The GenBank *Gallus gallus* mtGenome sequence, accession number NC_001323, was used to design, using the computer program Primer 3 (Rozen and Skaletsky, 1997), three of the four primer-pairs used in the experimental SNP analysis (**Table 2.1**). The fourth primer-pair was previously described by Sorenson et al. (1999) as a universal primer for amplifying mtDNA from most avian species. Together, the four primer pairs were expected to amplify PCR products of approximate total size of 8,876 bp. The FailSafe^{TM} PCR PreMixes (Epicentre, Madison, WI) were used to optimize the reaction conditions at primer annealing temperature of 56^{o}C. Following the optimization, PCR was carried out, the amplicons purified

and sequenced as previously described (Lin et at., 2006). The sequences were analyzed for SNPs using Phred, Phrap and Consed according to Gordon et al. (1998).

The *in silico* SNP analysis was carried out using completed whole mtGenome DNA sequences available in GenBank. A total of 11 sequences, each representing a unique chicken, was used in the ClustalW (Higgins et al., 1994) alignment to screen for SNPs. The accession numbers of the sequences used were AY235570, AP003580, AY235571, AP003318, AP003317, AP003319, AP003322, AP003323, NC001323, AB086102, and AP003321. A mismatch was considered a SNP only if at least 20 flanking nucleotides were of high quality and/or if the mismatch was observed in multiple individuals.

Statistical analysis

Haplotypes were displayed using Visual Haplotypes (http://pga.gs.washington.edu/VH1.html). Chi-square test was carried out to test if the distribution of haplotypes was in Hardy-Weinberg Equilibrium. Pairwise linkage disequilibrium between SNPs was done using GENEPOP (http://wbiomed.curtin.edu.au/genepop, Raymond and Rousset 1995).

2.4 Results and discussion

One hundred and thirteen SNPs were identified from the sequence analysis including 78 in the coding (**Figure 2.1**) and 35 in the non-coding (**Figure 2.2**) regions. A total of 91 SNPs were identified from the *in silico* analysis. From the 6,296 bp or about 40% of the mtGenome that were experimentally scanned for SNPs (**Table 2.1**), 41 SNPs were identified by the PCR-based resequencing (**Figure 2.3**). Nineteen SNPs observed in the *in silico* analysis were also observed in the experimental analysis, representing a validation of at least 25% of the virtual SNPs.

Among the 113 non-redundant SNPs observed in the mtGenome, 111 were substitutions and two were deletions. As expected, a disproportionate number of the SNPs were transitions. For example, in the coding region, 70 of the 78 SNPs or 91% were transitions of either C-T (41) or G-A (29) substitutions (**Figure 2.1**). Similarly, in the non-coding region, there was a significantly lower number of transversions: only one or 3% of the 35 SNPs observed in the D-loop was a G/C substitution (**Figure 2.2**). Among the 78 SNPs in the coding region, 19 were non-synonymous, 41 synonymous, and 18 in tRNA and rRNA (**Figure 2.1**).

The frequency of SNPs in non-coding and coding regions was 17.0 and 4.5 SNPs/1000 bp, respectively for the *in silico* analysis. The non-coding region and coding region SNP-frequency in the experimental analysis was 21.8 and 2.7 SNPs/1000 bp, respectively. These frequencies suggest, as expected, that the variation in D-loop is higher than in the coding region.

The results are consistent with previous results that the D-loop region is highly variable, making it a useful tool for phylogenetic analysis.

Eleven of the 17 haplotypes found in the coding region were from *in silico* analysis (**Figure 2.1**). Haplotype 1, one of the six observed haplotypes in the experimental analysis from the coding region, had the highest frequency (**Figure 2.4**). Haplotypes 1, 3, 4 occurred at a significantly higher frequency, together they were found in 88% of the birds evaluated (**Figure 2.4**). Chi-square test indicated that the distribution of haplotypes was not in Hardy-Weinberg Equilibrium ($X^2 = 17.8$, $P = 0.003$, **Figure 2.4**).

A total of 22 haplotypes was observed in the D-loop from both the *in silico* and experimental analysis (**Figure 2.2**). Eleven haplotypes were developed from the PCR-based resequencing. Haplotypes 1-9 were found only in broilers, and haplotypes 10 and 11 were only detected in White Leghorn chickens (**Figure 2.5**). Among the 9 broiler haplotypes, Haplotypes 1 and 2 occurred at a significantly higher frequency (**Figure 2.5**). Chi-square test of the 11 haplotypes indicated that the distribution of haplotypes was not in Hardy-Weinberg Equilibrium ($X^2 = 37.5$, $P < 0.001$, **Figure 2.5**).

About 42% of the pairwise linkage disequilibrium between the SNPs identified in the analysis in both broilers and White Leghorn chickens in D-loop were significant ($P < 0.05$, **Table 2.2**). Sixty-seven comparisons were highly significant ($P < 0.001$). In the coding region, however, only 6 out of 23 SNP comparisons were significant, with 4 of them highly significant (**Table 2.3**).

The SNPs described in the present work provide a foundation for which the role of the mitochondria in metabolic disorders could be further investigated. Some of the SNPs cause a change in amino acids which may affect important traits in the chicken. The SNPs in the non-coding region could also influence economic traits since the D-loop contains the origin of replication of the H-strand and the promoters for L and H-strand transcription. In humans and model species such as mice, common mtDNA polymorphisms have been associated with important disease traits as well as other characteristics including climatic adaptation and longevity (Wallace, 2005). It is our hope that the SNPs and haplotypes reported here will lead to similar investigations. Further association studies will thus be needed to show the value of our resources in mtDNA genotype:phenotype correlations in the chicken.

In the current study, haplotypes in the coding and non-coding regions were not in Hardy-Weinberg Equilibrium. Differences in the frequency of haplotypes may have been due to selection (Falconer and Mackay, 2002). Since cost limits the number of birds that could be analyzed, another explanation could be the relatively small number of birds evaluated. However, the haplotype frequency differences between egg- and meat-type chickens in the present work appear to be consistent with those reported by Niu et al. (2002).

Since mtDNA does not recombine (Berlin et al., 2003), mutations or SNPs in the mtGenome, especially those in coding regions, facilitate genotype:phenotype studies with important economic traits. Several human mitochondrial haplotypes have been shown to be associated with certain phenotypic traits, including disease, and individual responses to

medications and environmental factors. Haplotype J, for example, has been shown to occur in high frequency in centenarians. Association studies between mtDNA variation and longevity suggest that Haplotype J may be associated with tolerance to harmful effects of reactive oxygen species that may lead to the increased longevity (Chagnon et al., 1999; Esposito et al., 1999).

The data presented here represents, to our knowledge, the first investigation of variations across the entire chicken mtGenome. The results described could be useful for defining, as has been done in other animals, the molecular basis of many metabolic disorders, diseases, and abnormalities that affect chickens. For example, Moreno-Loshuertos et al. (2006) reported that the differences in reactive oxygen species production were correlated with mtGenome haplotypes in mouse. Using SNPs and haplotypes described by Johnson et al (2001) and Jenuth et al (1997), it was shown that variations in the dihydrouridine loop of tRNA arginine were associated with the production of hydrogen peroxide, a free radical, which influences respiratory performance. Therefore, although we did not carry out a phenotype:genotype association study, our novel SNPs and haplotypes may be useful in biochemical and molecular studies of economically important phenotypes in chicken. Additionally, though limited in scope and thus require further studies, the population differences in type and frequency of haplotypes may be useful in explaining genotype x environment interactions as well as age-related diseases and decline in performance in chickens.

Table 2.1. PCR primers and size of amplicons used in chicken mitochondrial (mt) genome haplotype analysis.

Primer ID	Primers [b]	Length (bp)[c]
mt1	F (L1732): 5' CTGGGATTAGATACCCCACTATGC 3' R (H3749): 5' ATAGCGGTTGCACCATTAGG 3'	2,017/1,710
mt2	F (L7764): 5' GTCGCCCACTTCCACTATGT 3' R (H10363): 5' AGCCCATGTAACGG TGACTC 3'	2,599/1,730
mt3[a]	F (L13040): 5' ATCC(A/G)TTGGTCTTAGGA(A/G)CCA3' R (H16064): 5' CTTCANT(C/T)TTTGG(C/T)TACAAG(A/G)CC 3'	3,024/1,620
D-loop	F (L1): 5' AGGACTACGGCTTGAAAAGC 3' R (H1236): 5' CATCTTGGCATCTTCAGTGCC3'	1,236/1,236

[a] Universal primers described by Sorenson et at. (1999). Alternative nucleotides are presented in parentheses.

[b] Primer binding-sites in the chicken mtGenome (GenBank Accession Number NC_001323) are presented in parentheses, L and H are light and heavy strands of mtDNA, respectively.

[c] Length in base pairs (bp) of the expected amplicon based on primer-binding sites. The number following / is the length of the sequence analyzed for single nucleotide polymorphisms.

Table 2.2. Statistical significance of the pairwise linkage disequilibrium (LD) analysis among the nucleotide variants in the D-loop of the chicken mitochondrial (mt) Genome.

Pos.[a]	54	164	196	207	209	214	222	236	239	240	243	253	258	278	307	312	338	354	360	364	414	443	683	788	848	855	1209
54	-	NS[b]	NS	NS	HS	0.050	NS	0.024	NS	0.003	HS	0.003	0.048	NS	0.003	HS	NS	NS	NS	NS	NS	0.050	NS	0.001	NS	NS	NS
164		-	0.010	HS[c]	NS	HS	HS	0.019	NS	HS	NS	HS	HS	NS	HS	NS	NS	NS	NS	NS	NS	HS	HS	NS	0.029	NS	0.035
196			-	0.046	NS	HS	0.011	NS	NS	0.002	NS	0.002	HS	NS	0.002	NS	NS	NS	NS	NS	NS	HS	0.010	NS	HS	NS	NS
207				-	NS	HS	HS	NS	NS	HS	NS	HS	HS	NS	HS	NS	NS	NS	NS	NS	NS	HS	HS	NS	0.005	NS	0.041
209					-	NS	NS	NS	NS	0.034	HS	0.036	NS	NS	0.036	HS	NS	NS	NS	NS	NS	NS	0.019	HS	NS	NS	NS
214						-	HS	NS	0.047	HS	NS	HS	HS	0.004	HS	NS	0.004	NS	0.004	0.003	NS	HS	HS	NS	0.043	NS	0.001
222							-	0.020	NS	HS	NS	HS	HS	NS	HS	NS	NS	NS	NS	NS	NS	HS	HS	NS	0.032	NS	0.036
236								-	NS	0.035	NS	0.035	NS	NS	0.034	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
239									-	0.038	NS	0.041	0.046	HS	0.043	NS	HS	NS	HS	HS	NS	0.049	NS	NS	NS	NS	NS
240										-	0.035	HS	HS	0.038	HS	0.035	0.037	NS	0.041	0.037	NS	HS	HS	NS	NS	NS	0.007
243											-	0.034	NS	NS	0.035	HS	NS	NS	NS	NS	NS	NS	0.019	HS	NS	NS	NS
253												-	HS	0.039	HS	0.034	0.039	NS	0.042	0.040	NS	HS	HS	NS	NS	NS	0.007
258													-	0.004	HS	NS	0.004	NS	0.004	0.004	NS	HS	HS	NS	0.045	NS	0.001
278														-	0.039	NS	HS	NS	HS	HS	NS	0.004	NS	NS	NS	NS	NS
307															-	0.036	0.038	NS	0.039	0.038	NS	HS	HS	NS	NS	NS	0.006
312																-	NS	NS	NS	NS	NS	NS	0.018	HS	NS	NS	NS
338																	-	NS	HS	HS	NS	0.004	NS	NS	NS	NS	NS
354																		-	NS	NS	NS	NS	NS	NS	NS	NS	NS
360																			-	HS	NS	0.004	NS	NS	NS	NS	NS
364																				-	NS	0.005	NS	NS	NS	NS	NS
414																					-	NS	NS	NS	NS	NS	NS
443																						-	NS	NS	0.046	NS	0.001
683																							-	0.040	NS	NS	0.014
788																								-	NS	NS	NS
848																									-	NS	0.018
855																										-	NS
1209																											-

[a] The position of each variant was determined in the GenBank chicken mtGenome sequence, NC_001323. LD was estimated using Genepop (Raymond and Rousset, 1995).

[b] The linkage disequilibrium was not significant ($P>0.05$);

[c] The linkage disequilibrium was highly significant ($P<0.001$).

Table 2.3. Statistical significance of the pairwise linkage disequilibrium (LD) analysis among the nucleotide variants in the coding regions of the chicken mitochondrial (mt) Genome.

Pos.[a]	2066	2204	2590	2673	3152	3264	7877	8070	8090	8183	8330	8464	9803	10072	10098	10219	10216	15435	15605	15683	15696	15865
2066	-	NS[b]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2204		-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.045	NS	NS	NS	NS
2590			-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2673				-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3152					-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3264						-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7877							-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8070								-	NS	NS	HS[c]	HS	NS	HS	NS	NS	NS	NS	NS	NS	NS	NS
8090									-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8183										-	NS	HS	NS	HS	NS	NS	NS	NS	NS	NS	NS	NS
8330											-	NS	NS	HS	NS	NS	NS	NS	NS	NS	NS	NS
8464												-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
9803													-	NS	NS	NS	NS	NS	NS	NS	NS	NS
10072														-	NS	NS	NS	NS	NS	NS	NS	NS
10098															-	NS	NS	NS	NS	NS	NS	NS
10219																-	NS	NS	NS	NS	NS	NS
10216																	-	NS	NS	NS	NS	0.046
15435																		-	NS	NS	NS	NS
15605																			-	NS	NS	NS
15683																				-	NS	NS
15696																					-	NS
15865																						-

[a] The position of each variant was determined in the GenBank chicken mtGenome sequence, NC_001323. LD was estimated using Genepop (Raymond and Rousset, 1995).

[b] The linkage disequilibrium was not significant ($P>0.05$);

[c] The linkage disequilibrium was highly significant ($P<0.001$)

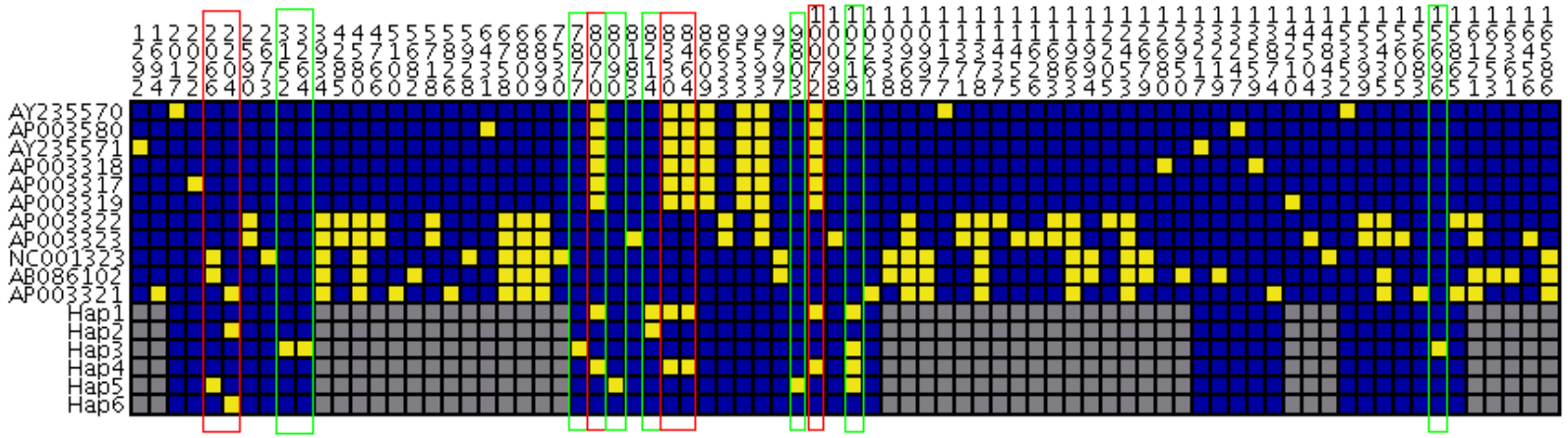


Figure 2.1. Visual Haplotypes-based view of haplotypes identified in the coding region of the mitochondrial genome (mtGenome). Blocks colored blue, yellow and gray represent common, rare alleles, and missing data, respectively.

Each single nucleotide polymorphism (SNP) position in the reference mtGenome sequence, accession number NC_001323, is shown by the vertical number. Horizontal labels consisting of two letters followed by 6 numbers represent accession numbers of sequences used from GenBank. Labels of Hap 1 to Hap 6 are sequences of the PCR amplicons produced by the primers described in **Table 2.1**. The red boxes indicate the SNPs found in both the *in silico* and experimental analyses; the green boxes show SNPs found only in the experimental analysis.

The SNPs, with the common allele listed first, and the positions in the mtGenome at which they occur are:

T/C: 1292^{R}, 2204^{R}, 2673^{R}, 4285^{NS}, 5100^{R}, 5826^{NS}, 6431^{R}, 9803^{S}, 10219^{S}, 11378^{S}, 11963^{S}, 13219^{NS}, 13559^{S}, 14504^{S}, 14843^{S}, 15399^{S}, 15435^{S};

C/T: 2066^{R}, 3934^{R}, 6758^{S}, 6800^{S}, 8070^{NS}, 8214^{R}, 8330^{R}, 8464^{NS}, 8609^{S}, 8633^{S}, 10338^{S}, 10968^{S}, 10997^{S}, 11437^{NS}, 11455^{NS}, 11626^{S}, 11994^{S}, 12453^{S}, 12679^{S}, 15605^{NS}, 15865^{S}, 16253^{S}, 16361^{S}, 16456^{NS};

A/G: 4580^{S}, 4766^{S}, 8090^{S}, 9593^{S}, 9797^{S}, 11177^{R}, 11683^{S}, 12205^{S}, 13217^{S}, 13874^{S}, 14210^{S}, 15352^{NS}, 15696^{S};

G/A: 2017^{R}, 2022^{R}, 2590^{R}, 3152^{R}, 6899^{S}, 7877^{NS}, 8183^{S}, 9533^{S}, 10072^{S}, 10098^{NS}, 10261^{S}, 12680^{NS}, 12950^{R}, 13247^{S}, 16121^{R}, 16586^{S};

T/G: 5682^{NS}, 5718^{NS}, 11371^{NS};

A/C: 5928^{NS};

G/C: 7530^{NS};

T/A: 15683^{NS};

C/A: 1694^{R}, 3264^{R}.

Superscripts NS, R, S indicate non-synonymous, RNA, and synonymous SNPs, respectively.

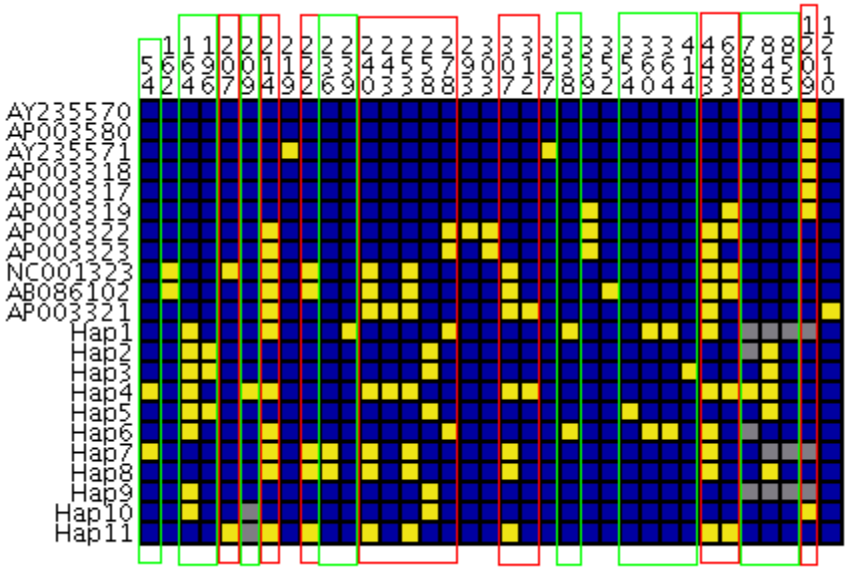


Figure 2.2. Visual Haplotypes-based view of haplotypes identified in the non-coding region of the mitochondrial genome (mtGenome).

Blocks colored blue, yellow and gray represent common, rare alleles, and missing data, respectively.

Each single nucleotide polymorphism (SNP) position in the reference mtGenome sequence, accession number NC_001323, is shown by the vertical number. Horizontal labels consisting of two letters followed by 6 numbers represent accession numbers of sequences used from GenBank. Labels of Hap 1 to Hap 11 are sequences of the PCR amplicons produced by the primers described in **Table 2.1**.

The red boxes indicate the SNPs found in both the *in silico* and experimental analyses; the green boxes show SNPs found only in the experimental analysis.

The SNPs, with the common allele listed first, and the positions in the mtGenome at which they occur are:

T/C: 162, 164, 196, 258, 303, 307, 352, 364, 443, 1209;

C/T: 207, 214, 222, 240, 243, 253, 293, 312, 327, 360, 414;

A/G: 219, 236, 278, 329, 338, 354;

G/A: 209, 239, 683, 786, 1210;

G/C: 54,

C/deletion: 848, 855.

Figure 2.3. Consed alignment showing segregation of a single nucleotide polymorphism

Consed alignment showing segregation of a C-T single nucleotide polymorphism at position 8,070 (GenBank Accession Number NC_001323) in commercial chicken populations.

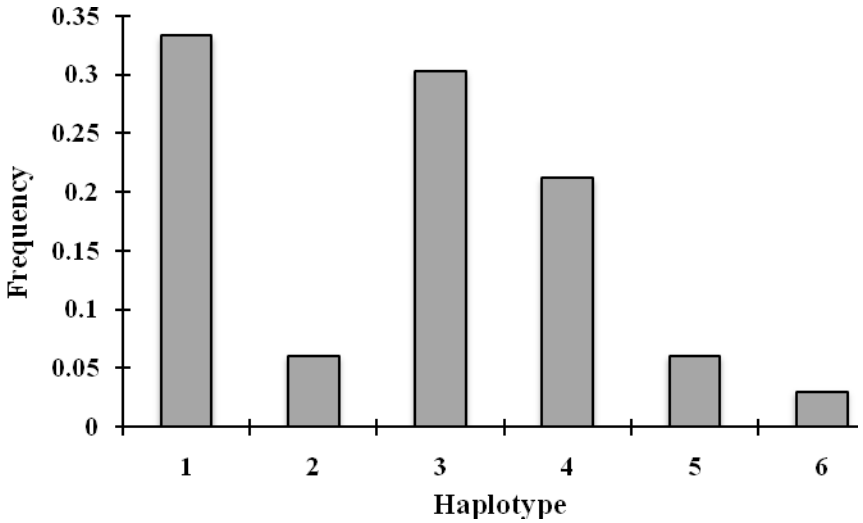


Figure 2.4. Frequency distribution of coding region mitochondrial DNA haplotypes in broilers.

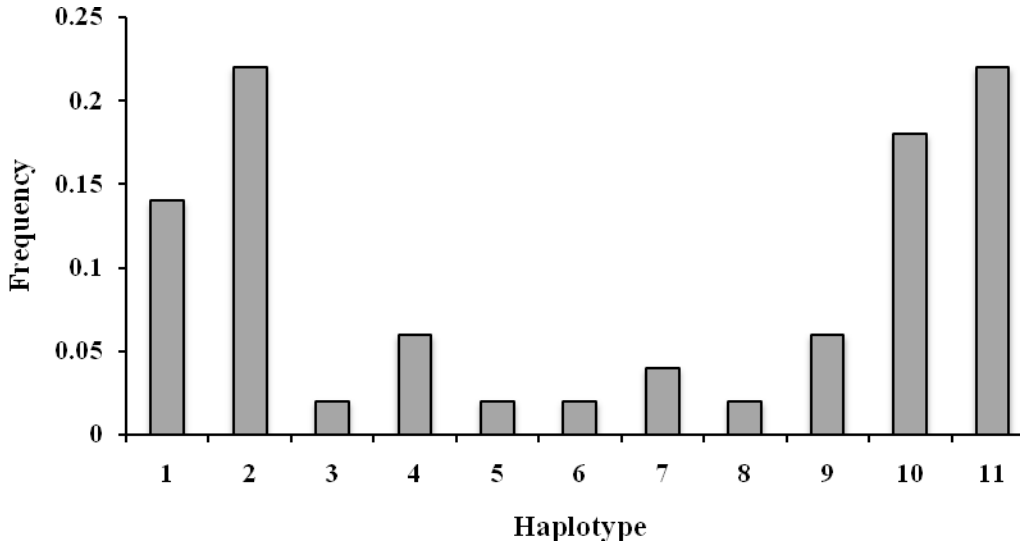


Figure 2.5. Frequency distribution of non-coding region mitochondrial DNA haplotypes in commercial broiler and White Leghorn populations.

Haplotypes 1-9 were only found in broilers, while Haplotypes 10 and 11 were only detected in White Leghorn chicken.

CHAPTER 3

The Mitochondrial Genome Sequence and Molecular Phylogeny of the Turkey, *Meleagris gallopavo*

3.1 Abstract

To test the hypothesis of the dissertation that free radical damage of the mitochondrial geome (mtGenome) of birds affects longevity, the turkey (*Meleagris gallopavo*) mtGenome, which was not publicly available, was needed. Here, we used PCR-based methods with 19 pairs of primers designed from the chicken and other species to develop a complete turkey mtGenome sequence. Two of the primer pairs were then used to screen for single nucleotide polymorphisms (SNPs) in the D-loop and 16S rRNA in heritage turkey varieties. The SNPs and the whole genome sequence were used to assess the genetic relatedness among heritage turkeys and between the turkey and 56 avian species whose mtGenome sequences were publicly available. A total length of 16,967 bp of the turkey mtGenome was obtained, with 85% similarity to chicken mtGenome. In addition to the 13 genes and 24 RNA (22 tRNA and 2 rRNA) found in all mtGenomes, the sequence developed here also contained a one base pair insertion in ND3 that is found in most birds including different Gallus species. Among the SNPs identified and validated from comparing heritage turkey varieties, one was found only in the Spanish Black and Blue Slate. Phylogenetic analysis based on the SNPs showed that among turkey varieties, Narragansett and Royal Palm are more closely related, and the commercial turkeys are closest to Blue Slate and Spanish Black. Further, mtGenome-based phylogenetic analysis suggests that the turkey is most closely related to the chicken, *Gallus gallus*, and quail, *Corturnix japonica*. In addition to

providing support for hybridization and microsatellite-defined relationships, the sequence and SNPs will be useful in assigning function to the mtGenome.

Keyword: turkeys, mitochondrial genome sequence, phylogenetic relationships

3.2 Introduction

The turkey, *Meleagris gallopavo*, is native to North America and exists widely as both an important wild bird and the second most important poultry meat species. There have been both intentional and non-intentional crossbreeding between wild and domesticated turkeys (Aldrich, 1967). The domesticated turkey, derived by crossbreeding and line breeding is characterized as a single breed with 8 distinct varieties (Kennamer et al., 1992). The varieties are Black, Bronze, Narragansett, Slate, Beltsville Small White, Bourbon Red, Royal Palm and White Holland (Austic and Nesheim, 1990). Commercial turkeys raised for meat are believed to be derived from the White Holland and the Bronze varieties through a combination of mating and selection (Austic and Nesheim, 1990).

Differences and similarities in size and plumage coloration among the varieties were the main criteria for the characterization (Aldrich, 1967). Thus, it remains unclear whether these varieties have additional differences in other phenotypes including biochemical traits and if the differences can be detected at the molecular level. Recently, however, attempts have been made to further evaluate differences among turkey varieties at the phenotypic, molecular, and biochemical levels. For example, Hartman et al. (2006) reported differences among turkey varieties in plasma uric acid, a biochemical marker that has been used as an indicator of oxidative stress levels in animals. In unpublished work described in his MS thesis, Gyenai (2006), showed that the turkey varieties differed in susceptibility to a dose of furazolidone that induces dilated cardiomyopathy.

Differences among the turkey varieties have also been investigated at the molecular level. Smith et al. (2005a) and Kamara et al. (2007) used different DNA marker systems, including randomly amplified polymorphic DNA, microsatellite, and single nucleotide polymorphisms

(SNPs) to establish that the varieties are distinct at the molecular level. These marker systems were nuclear-based and thus differ from mitochondrial DNA in rate of sequence evolution. Therefore, to further our understanding of the genetic relatedness among these varieties, mitochondrial DNA analysis is essential.

Unlike non-commercial domesticated turkeys, wild turkeys, have been extensively evaluated both at the phenotypic and molecular level. Using amplified fragment length polymorphism markers and the mitochondrial control region derived using heterologus primers from the chicken, Mock et al. (2002) evaluated genetic variation among turkeys distributed widely in the United States of America. The molecular analyses of the varieties revealed relationships among turkeys from distinct geographic regions that were also consistent with earlier morphological designation of subspecies. Because of the lack of a public turkey mitochondrial DNA sequence, Szalanski et al. (2000) also used heterologous primers from the chicken and quail to evaluate the extent of genetic variation and differentiation in the turkey and its six known subspecies.

The phylogenetic relationship between the turkey and other avian species, especially gallinaceous birds, is also very little understood. This paucity is probably due to the lack of a publicly available turkey mtGenome sequence that could provide an opportunity to use non-recombining sequences with diverse rates of evolution for turkey phylogenetics. The few phylogenetic studies that have included the turkey includes that by Helm-Bychowski and Wilson (1986), which used restriction enzyme-based maps involving 161 restriction sites to describe relationships among the turkey and six other phasianoids that were different from those by traditional and protein-based comparisons. Using allozymes, Randi et al. (1991) grouped phasianoids into two main categories, one including the turkey and the other the quails, with the

guineafowl distantly related to both groups. Using DNA-DNA hybridization, Sibley and Ahlquist (1990) reported in broad terms relationships among galliform birds including chicken, guineafowl, quail, and turkey. Because of the lack of consensus of the relationships among galliform birds, especially among the chicken, quail, and turkey, phylogenetics studies of these poultry species continue. In a recent study, Kaiser et al. (2007) reported that based on insertion events of *CR1* retrotransposable elements, the turkey was more closely related to quail and chicken but distant to the guineafowl. Despite the use of these diverse genetic markers, our understanding of galliform relationships continues to be marginal and the phylogenies continue to be without congruence. The mtGenome sequence of the turkey will provide access to genes with different evolutionary rates than those that have been used to assess galliform phylogenies.

Since the mtGenome is considered a very reliable and thus very widely used phylogenetic tool (Avise, 1994), it is surprising that the turkey, an ecologically and agriculturally important species, lacks a whole mtGenome sequence. In addition to its utility for phylogeny of galliform birds, the sequence of the turkey mtGenome could be a useful tool for establishing the influence of the mitochondrion and its genes on economically important phenotypes. Here, the primary objective was to develop and annotate the turkey mtGenome which will form a foundation for mutation analysis to test the hypothesis of the dissertation that free radical damage of the mtGenome of birds is associated with longevity.

3.3 Materials and methods

DNA samples

Birds used for the variation and phylogenetic analysis included heritage turkeys obtained from a commercial breeder (Privett Hatcheries, New Mexico). The birds from which feather pulp was obtained belonged to Narragansett (N), Royal Palm (RP), Blue Slate (BS) or Spanish Black (SB) turkey varieties. Additionally, birds from a non-heritage commercial turkey from British United Turkeys (BUT) wee also used. Pulp was obtained from secondary feathers collected from each bird and used to isolate genomic DNA using a minor modification of the recommended protocol for the DNeasy Tissue Kit (Qiagen, Valencia, CA). The modification included the addition of 100 mg/ml dithiothreitol solution to facilitate lysis of the feather pulp during incubation.

Mitochondrial genome sequencing

The turkey mtGenome sequence was developed from sequencing of PCR products or amplicons obtained using heterologous primers. The primers included universal oligos previously described by Sorenson et al. (1999) and those developed for the present work (VT Primers) using *Gallus gallus* mtGenome DNA sequence in GenBank, accession number NC_001323. In addition to the conventional criteria for selecting primers, oligos were designed and chosen by Primer 3 (Rozen and Skaletsky, 1997) for their ability to produce overlapping amplicons of 2 to 4 kb. Primers were optimized at an annealing temperature of 56^{o}C using the FailSafe^{TM} PCR PreMixes kit according to the manufacturer's recommendation (Epicentre Technology, Madison, WI). Following optimization, the successful premix that produced a single amplicon was used to carry out PCR for the specific primer pair. For each primer-pair, at

least two independent PCR products were purified and sequenced using both reverse and forward primers as previously described (Lin et al., 2006). Internal primers were also developed to complete the sequencing of some long-range PCR products. The internal primers also ensured that some regions of the turkey mtGenome were sequenced at least three times including in those in the regions were two or more primers produced overlapping amplicons. The sequences were assembled using a combination of bioinformatics tools including of Phred, Phrap and Consed (Gordon et al., 1998).

Sequence validation and annotation

The whole genome sequence was validated at two levels: multiple sequencing of each region and sequence comparison with GenBank mtGenome sequences from other birds. An additional validation of the turkey mitochondrial DNA sequences was based on sequence similarity as revealed by a ClustalW (Higgins et al., 1994) based multi-alignment using mitochondrial DNA sequences publicly available for *Coturnix japonica (*accession number NC_003408*), Numida meleagris (*accession number NC_006308*), and Gallus gallus*. To annotate the sequence, BLAST 2 (Tatusova and Madden., 1999) and GeneDoc (Nicholas et al., 1997) were used to compare the assembled sequence to database mtGenome sequences. Additionally, ORF-Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and vsfold4 (http://www.rna.itchiba.ac. jp/~vsfold/vsfold4/), a DNA sequence viewer and annotation tool and RNA secondary structure prediction program, respectively, were also used to further annotate and/or validate the BLAST 2 and GeneDoc annotation of the turkey mitochondrial DNA sequence.

Phylogenetic analysis

Two levels of phylogenetic studies were carried out: among the turkey varieties described above using variation in the D-loop and 16S rRNA, and among avian species using the new turkey sequence and whole genome mitochondrial sequences available in GenBank. To carry out the among varieties phylogenetic analysis, a total of 30 birds, 6 from each of BS, N, RP, SB, and BUT populations were used to search for sequence variants within the D-loop and 16S rRNA. The newly developed turkey mitochondrial DNA sequence was used to design primers using the web based program Primer 3 (Rozen and Skaletsky, 1997). The primers developed and used for PCR as described above were TDLF: 5'-CCAAGGATTACGGCTTGAAA-3', TDLR: 5'-TTAAGCTATGGGGGCTGTTG-3', from the D-loop and T16SF: 5'-ACAACCAAGCA AAGCGAACT-3', and T16SR: 5'-ATGGGCTCTTGGAGGAGATT-3', from the 16S rRNA. The sequences generated from the amplicons were analyzed for SNPs using Phred, Phrap and Consed as previously described (Lin et al., 2006). The multiple sequence alignment was obtained by using ClustalX (Thompson et al., 1997). Phylogenetic analysis was conducted by PAUP* version 4.0 (Swofford, 2002). The *Gallus gallus* sequences were used as outgroups for rooting. Phylogenetic trees were constructed using minimum evolution, neighbor-joining, maximum parsimony, and maximum likelihood methods. One thousand bootstrap replicates were used to assess the confidence in the grouping in minimum evolution, neighbor-joining, and maximum parsimony methods (Felsenstein and Kishino, 1993). To select an appropriate model for maximum likelihood analysis, we used Modeltest 3.8 (Posada, 2006). Using the Akaike information criterion, the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with equal rates for all sites was selected.

Using the rationale that different segments of the mtGenome undergo varying rates of evolution, the phylogenetic analyses among species were based on the whole genome sequence, 16S rRNA, and the coding region that included 12 protein-coding genes but not ND6 which is encoded on a different strand, two rRNA genes and 19 tRNA genes. A total of 57 species, including the turkey, chicken, quail, and guineafowl were used (**Table 3.3**). In the 16S rRNA-based phylogenetic analysis, the American alligator and human sequences were used as outgroups for rooting. Based on the results of 16S rRNA phylogenetic analysis, Anseriformes were selected as outgroup for the coding region-based phylogentic analysis. ClustalX (Thompson et al., 1997) was also used for the multiple sequence alignment. Phylogenetic analysis, tree construction, model selection and statistical tests were as described above. One thousand bootstrap replicates were used to assess the confidence in the grouping in minimum evolution, neighbor-joining, and maximum parsimony methods (Felsenstein and Kishino, 1993). Again, using the Akaike information criterion as the basis for selecting models for the 16S rRNA and coding region phylogenetic analyses, the General Time Reversible + Gamma + Proportion Invariant (GTR+G+I) model of evolution were selected with gamma distribution shape parameter of 0.6002 and 0.9201, respectively.

3.4 Results and discussion

All but four primer-pairs used to generate amplicons that were sequenced are new (**Table 3.1**). From the amplicons, a total sequence length of 16,967 bp, representing the turkey mtGenome was developed, validated, and annotated. The sequence has been submitted to GenBank and assigned accession number EF_153719. The sequence showed 85, 84 and 83% average similarity with the chicken, Japanese quail and guineafowl mtGenome sequences in GenBank, respectively. The turkey mtGenome sequence was longer than that of the chicken, Japanese quail and guineafowl mtGenomes that are 16,775, 16,697 and 16,726 bp, respectively. The difference in length is due to the longer D-loop in the turkey, 1,413 bp compared to about 1,200 in the chicken, quail, and guineafowl. This is consistent with observations in diverse birds as the D-loop is reported to vary from 1,227 bp in *Gallus gallus* to 2,715 bp in the common buzzard, *Buteo buteo*.

Within the turkey mtGenome sequence, the most frequent nucleotide in the H-strand was A, followed by C, G and T. In the chicken and human mtGenomes, the most frequent nucleotide is a C, followed by A, T, and G while in the mouse the order of frequency is an A followed by T, C, and G. The GC content of 43.2% observed in the turkey sequence is also consistent with that reported in other birds ranging from *Apteryx hastii* to *Aythya Americana* with 42 and 48%, respectively. These values are much higher than for other vertebrates including the mouse for which the average GC content has been reported to be about 36%. Though the GC content of the turkey mtGenome appears to be consistent with the average in vertebrates, it is lower than the chicken (46%) and goose (47%).

Thirteen protein coding genes and 24 RNA (22 tRNA and 2 rRNA) were identified within the turkey mtGenome sequence (**Table 3.2**). While 12 of the protein coding genes were

located on the H strand, the gene for NADH dehydrogenase subunit 6 (ND6) was on the L-strand. On the other hand, while the sequences for two rRNAs (12S and 16S) were on the H-strand, those for 9 tRNAs were located on the L-strand. These results are consistent with the mtGenome organization in other vertebrate species.

Finally, another characteristic of the turkey mtGenome sequence was an extra base at position 174 of the gene for NADH dehydrogenase subunit 3 (ND3). This additional nucleotide has been reported in 46 other birds (Mindell et al., 1998). It has been suggested that the additional nucleotide causes a reading frame change which results in multiple stop codons in the ND3 gene sequence. However, since the frame shifting does not affect ND3 function, Mindell et al. (1998) hypothesized that birds might have, as yet unknown, a mechanism such as translational frame shifting or RNA editing to correct this anomaly.

A total of three SNPs, two in the 16S rRNA at nucleotide positions 1,623 (A-C) and 1,951 (A-T), and one in the D-loop at position 15,953 (C-T), were detected (**Figure 3.1, Table 3.4**). The two SNPs in 16S rRNA formed three haplotypes AA, CA, and CT, respectively. The CA haplotype was the most frequent in the turkey populations evaluated, ranging from 50% in the RP to 100% in BS, SB, N, and BUT populations. In the RP, the haplotypes AA and CT had a frequency of 0.20 and 0.30, respectively. While the C allele of the SNP in the D-loop was detected only in the BS and SB varieties, the T allele was observed only in the N, RP, and BUT birds. Given the low level of variation within the 16S rRNA and D-loop within the heritage and BUT turkeys, it is not surprising the relationships among the varieties appear to be very close (**Figures 3.2**). The D-loop based phylogenetic analysis suggests a closer relationship between N and RP and among the BUT, BS and SB varieties (**Figure 3.2**). Similarly, the 16S rRNA-based

analyses showed that RP was distant from all the other varieties, which were on average more closely related to each other (**Figure 3.3**).

Fifty-six whole mtGenome sequences available in GenBank used to determine the relatedness of the turkey to other birds, especially Galliformes, included diverse birds ranging from *Alectura lathami* to *Vidua chalybeate* (**Table 3.3**). The between species 16S rRNA-based minimum evolution, maximum likelihood, maximum parsimony, and neighbor-joining trees which were congruent, showed a closer relationship between the turkey and chicken and quail but relatively more distant to the guineafowl, also a gallinaceous bird (**Figure 3.4**). Another Galliformes, *Alectura lathami*, also showed a closer relationship to the turkey (**Figure 3.4**, Kimball et al., 1999). Many of the relationships shown by the analyses including among the Gallus species and among the Anseriformes appear to be consistent with published taxonomy (Sibley and Ahlquist, 1990).

The relationships defined by phylogenetic analyses involving 12 protein-coding genes, two rRNA genes and 19 tRNA genes were consistent with those shown by the 16S rRNA (**Figure 3.5).** The relationships among turkey, chickens (the multiple Gallus species) and quails (Corturnix) were similar and with higher bootstrap values. As revealed in the 16S rRNA tree, *Alectura lathami* was also shown to be closely related to the turkey-jungle fowl-guineafowl branch while the *Buteo buteo*, *Smithornis sharpie*, *strigops habroptilus*, *Larus dominicanus* branch was most distant (Gibb et al., 2007).

The animal mtGenome is generally considered to be under selection for both small size and a conserved gene order (Rand and Harrison, 1986; Quinn and Wilson, 1993; Boore, 1999). Animal mitochondrial genomes rarely contain either introns or intergenic spacers (Quinn and Wilson, 1993, McKnight and Shaffer, 1997). The turkey, an important agricultural and model

avian species remains one of the least understood phylogenetically and one of many birds for which there is no publicly available whole mtGenome DNA sequence. Here, we have described the turkey mtGenome sequence and showed that it is similar in length and nucleotide composition to that of most other birds. Compared to mammalian species, only a limited number of birds, which generally exceed mammals in the total number of species, have had their whole mtGenome sequenced. With the sequence described here, the total currently available in public databases including GenBank is at 57. While the mtGenome gene content and gene order is remarkably stable across vertebrate species, the avian species are an exception to this stability. Several gene order rearrangements have occurred in avian mtGenomes, primarily affecting the area around the ND6 gene, and the avian species have several unusual features in their mitochondrial DNA, such as the lack of the traditional origin of replication for the light strand and the as-yet unidentified splicing function to repair the insertion found in the ND3 gene in most avian mtGenomes. For these reasons it is particularly important in avian species to sequence the entire mtGenome, not just individual genes. Like in most avian species, in the turkey mtGenome cytochrome b is followed by ND6 and the tRNA-Glu genes. This structural arrangement is, however, different from those of mammalian (and other vertebrate) mtGenomes. In mammals the genes for ND6 and tRNA-Glu are located between ND5 and cytochrome b (Desjardins and Morals, 1990).

Two portions of the mtGenome sequence normally reported to be under different rates of divergence in animal mitochondria were used to evaluate the genetic relatedness among turkey varieties, which remain very little understood. The analyses of the genetic relatedness among the turkey varieties using sequence divergence within 16S rRNA and the D-loop appear to provide molecular support for a long-held view (American Standard of Perfection, 2001), arising

primarily from morphological data, that turkeys are a single breed. The very low level of variation in the D-loop and 16S rRNA suggest that unlike the chicken (Niu et al., 2002) and other animals including the horse for which 31 base substitutions were observed in only 397 bp of sequences compared (Bowling et al., 2000), existing subpopulations or varieties are more closely related. The relationships defined in the current work appear to be consistent with previous report by Smith et al. (2005a) that was based on nuclear DNA markers which showed that RP is more closely related to N but distant from BS and SB turkey varieties. Additionally, recent microsatellite data described by Kamara et al (2007) show close relationships among BS N, RP and SB varieties though RP was more closely related to SB than to the other varieties as well as the BUT.

The new sequence was also used to evaluate the phylogenetic relationships between the turkey and other birds. To date, the most extensive comparisons of Galliformes have involved partial sequences from mitochondrial (Dimcheff et al., 2002) and nuclear genes (Smith et al., 2005b; Kaiser et al., 2007) as well as the hybridization results of Sibley and Ahlquist (1990). The relationships between the turkey and other Galliformes were in general agreement with Sibley and Ahlquist (1990), Smith et al (2005b) and Dimcheff et al. (2000, 2002). Briefly, these studies showed that the turkey as a sister to clades containing *Gallus* and *Coturnix* species. Further, and as observed here, *Numida* was basal to the *Coturnix* and *Gallus* clades but more distant to the turkey. The high bootstrap values provide strong support for the relationships defined here between the turkey and other Galliformes.

In summary, the turkey mtGenome sequence was developed and used to evaluate the genetic relatedness among varieties and between the turkey and other birds. Though our analyses of relationships among birds were limited in the extent of statistical parameters included, the

mtGenome sequence provides a resource for extensive phylogenetic analyses. Since the evolutionary relationships among Galliformes continues to be without a general consensus, the whole mtGenome sequence described here will provide an additional tool for generating more data needed to understand the turkey's relationship with other gallinaceous birds. The sequence will also facilitate assignment of function to the mtGenome especially the role of mitochondrial genes in variation in economically important phenotypes in the turkey. While such reports are very few in birds and other livestock species, an argument has been made that mutations in the mitochondria can be blamed for many diseases and abnormalities in human (Wallace, 2005). In the chicken, a suggestive association between variation in the mtGenome and some phenotypes was previously reported by Li et al. (1998). The SNPs identified in the turkey mtGenome, though limited, as well as the sequence provide a foundation to begin to more widely evaluate this important organelle in the turkey.

Table 3.1. Sequences of primers used in the polymerase chain reaction.

Primer ID	Primer sequence
TL1F[a]	AARCCMGAATGRTAYTTYCTWTTYGC
TL1R[a]	GTGGCTGGCACARGATTTACC
TL1in1F	AACCCGCGTACAAGCTCTAA
TL1in1R	TCTTCAGTGCCATGCTTTTG
TL1in2F	TCCTACCCCCAACATCCATA
TL1in2R	GCTTAAGGTTAATTACTGCTGAATACC
TL2F[a]	YAAAGCATGRCACTGAA
TL2R[a]	TYTCAGGYGTARGCTGARTGCTT
TL3Fnew	GCCCTTGGAAGGAGGATTTA
TL3Rnew	CAGTTCTGCACGGATTAGCA
TL3in1F[a]	CAACCGTACCGTAAGGGAAA
TL3in1R	CGTCTGGTTTGCACTCAGAA
TL3in2F	AGCCCCCTCGAAAAAGAATA
TL3in2R	AGGCCGGCTAGAGATAGGAG
TL3in3F	GTGTTCTCGTGCAAAAACGA
TL3in3R	GGTGGTGGGATTTTGAGATG
TL3in4F	CTCGGCAAATGCAAAAGACT
TL3in4R	TGGGAGGTTCAGGAAACTTG
TL4F[a]	CCYCTGTAAAAAGGWCTACAGCC
TL4in2F	CATAAAACCCCCAGCACTGT
TL4Rnew2	TAATTTGCTGGGTCGAAACC
TL4in3F	TGGAGGTCTTACGGGAATTG
TL4in3R	GGGTTGTTTGAGCGAGAAGA
TL4in4F	GAAGGAATCGAACCCTCACA
TL4in4R	CTGCTTTCGGTTTCCTTCTG
TL4in5F	GCCTGATCCTCCCTCCTATC
TL4in5R	ATGTCCGGCTGTAAGGTTTG
TL5Fnew	CAAACAACCCCAGACACAGA
TL5Rnew	GGCTGAGTAGGAAGGCAGTTT
TL5in1F	AAAACCAAACCCCATCCTTC
TL5in1R	GGGTTGTAGGCCTCGTGTAA
TL6F[a]	ATCCRTTGGTCTTAGGARCCA
TL6R[a]	CTTCANTYTTTGGYTTACAAGRCC
TL6in1F	ACAAGCAATCCAACCAGACC

TL6in1R	GTTTGGGATTGAGCGTAGGA
TL6in2F	TCCGCATGACACTGCTAGTC
TL6in2R	GATGAAGAAGAATGAGGCGC

[a] Universal primers described by (Sorenson et al., 1999) were also used.

Table 3.2. Sequence annotation of the mitochondrial genome of the turkey, *Meleagris gallopavo.*

Genes	Location	Size (bp)	Initial Codon	Terminal Codon
tRNA-Phe	1-67	67		
12S ribosomal RNA (12S rRNA)	68-1040	972		
tRNA-Val	1041-1113	73		
16S ribosomal RNA (16S rRNA)	1114-2731	1618		
tRNA-Leu	2732-2805	74		
NADH dehydrogenase subunit 1 (*ND1*)	2821-3795	975	ATG	TAA
tRNA-Ile	3796-3867	72		
tRNA-Gln	3876-3945[a]	70		
tRNA-Met	3945-4013	69		
NADH dehydrogenase subunit 2 (*ND2*)	4014-5054	1041	ATG	TAG
tRNA-Trp	5054-5131	78		
tRNA-Ala	5138-5205[a]	68		
tRNA-Asn	5209-5280[a]	72		
tRNA-Cys	5283-5347[a]	65		
tRNA-Tyr	5347-5417[a]	71		
Cytochrome oxidase subunit 1 (*COX1*)	5419-6969	1551	GTG	AGG
tRNA-Ser	6962-7035[a]	74		
tRNA-Asp	7038-7106	69		
Cytochrome oxidase subunit 2 (*COX2*)	7108-7791	684	ATG	TAA
tRNA-Lys	7793-7861	69		
ATPase subunit 8 (*ATPase8*)	7863-8027	165	ATG	TAA
ATPase subunit 6 (*ATPase6*)	8018-8701	684	ATG	TAA
Cytochrome oxidase subunit 3 (*COX3*)	8701-9487	787	ATG	TGC
tRNA-Gly	9486-9553	68		
NADH dehydrogenase subunit 3 (*ND3*)	9554-9905	352	ATG	TAA
tRNA-Arg	9907-9974	68		
NADH dehydrogenase subunit 4 light-chain (*ND4L*)	9975-10271	297	ATG	TAA
NADH dehydrogenase subunit 4 (*ND4*)	10265-11645	1381	ATG	TGC
tRNA-His	11643-11711	69		
tRNA-Ser	11713-11777	65		
tRNA-Leu	11779-11849	71		
NADH dehydrogenase subunit 5 (*ND5*)	11850-13667	1818	ATG	TAA
Cytochrome b (*Cytb*)	13671-14813	1143	ATG	TAA
tRNA-Thr	14816-14884	69		
tRNA-Pro	14885-14956[a]	72		
NADH dehydrogenase subunit 6 (*ND6*)	14964-15484[a]	521	ATG	TAG
tRNA-Glu	15486-15553[a]	68		

[a] Coded on the complementary (L) strand.

Table 3.3. Scientific and common names of birds for which whole mitochondrial genome sequences are available in GenBank.

Bird	Accession	Common name
Alectura lathami	NC_007227	Australian brush-turkey
Alligator mississippiensis [b]	NC_001922	American alligator
Anser albifrons	NC_004539	White-fronted goose
Anseranas semipalmata	NC_005933	Magpie goose
Apteryx haastii	NC_002782	Great spotted kiwi
Apus apus	NC_008540	Swift
Ardea novaehollandiae	NC_008551	White-faced heron
Arenaria interpres	NC_003712	Ruddy turnstone
Aythya americana	NC_000877	Redhead
Branta canadensis	NC_007011	Canada goose
Buteo buteo	NC_003128	Common buzzard
Casuarius casuarius	NC_002778	Southern cassowary
Cathartes aura	NC_007628	Turkey vulture
Ciconia boyciana	NC_002196	Oriental stork
Ciconia ciconia	NC_002197	White stork
Cnemotriccus fuscatus	NC_007975	Fuscous flycatcher
Corvus frugilegus	NC_002069	Rook
Coturnix chinensis	NC_004575	Blue-breasted quail
Coturnix japonica	NC_003408	Japanese quail
Cygnus columbianus	NC_007691	Tundra swan
Diomedea melanophris	NC_007172	Black-browed albatross
Dromaius novaehollandiae	NC_002784	Emu
Dryocopus pileatus	NC_008546	Pileated woodpecker
Eudromia elegans	NC_002772	Elegant crested-tinamou
Eudyptes chrysocome	NC_008138	Rockhopper penguin
Eudyptula minor	NC_004538	Little blue penguin
Falco peregrinus	NC_000878	Peregrine falcon
Falco sparverius	NC_008547	American kestrel
Gallus gallus	NC_001323	Chicken
Gallus lafayettei	NC_007239	Ceylon junglefowl
Gallus sonneratii	NC_007240	Gray junglefowl
Gallus varius	NC_007238	Green junglefowl
Gavia pacifica	NC_008139	Pacific loon
Gavia stellata	NC_007007	Red-throated loon
Haematopus ater	NC_003713	Blackish oystercatcher
Homo sapiens [b]	NC_001807	Human
Larus dominicanus	NC_007006	Southern black-backed gull
Menura novaehollandiae	NC_007883	Superb lyrebird
Meleagris gallopavo [a]	**EF_153719**	Domestic turkey
Micrastur gilvicollis	NC_008548	Lined forest-falcon
Ninox novaeseelandiae	NC_005932	Morepork
Nipponia nippon	NC_008132	Crested ibis
Numida meleagris	NC_006382	Helmeted guineafowl
Pandion haliaetus	NC_008550	Osprey
Phaethon rubricauda	NC_007979	Red-tailed tropicbird

Podiceps cristatus	NC_008140	Great crested grebe
Pterocnemia pennata	NC_002783	Lesser rhea
Pterodroma brevirostris	NC_007174	Kerguelen petrel
Pteroglossus azara flavirostris	NC_008549	Ivory billed aracari
Rhea americana	NC_000846	Greater rhea
Smithornis sharpei	NC_000879	Grey-headed broadbill
Spizaetus alboniger	NC_007599	Blyth's hawk-eagle
Spizaetus nipalensis	NC_007598	Mountain hawk-eagle
Strigops habroptilus	NC_005931	Kakapo
Struthio camelus	NC_002785	Ostrich
Synthliboramphus antiquus	NC_007978	Ancient murrelet
Taeniopygia guttata	NC_007897	Zebra finch
Tinamus major	NC_002781	Great tinamou
Vidua chalybeata	NC_000880	Steelblue widowfinch

[a] The sequence developed in this work.
[b] Species used as outgroup.

Table 3.4. The frequency estimates of the 16S rRNA haplotypes within the heritage varieties.

Haplotype[a]	RP (10)[b]	BS (11)	SB (7)	N (8)	BUT (7)
-A-A-	2	0	0	0	0
-C-A-	5	11	7	8	7
-C-T-	3	0	0	0	0

[a] SNP position at 1,623 and 1,951 according to the turkey mitochondrial genome (GenBank accession number: EF_153719).

[b] Numbers of turkeys used in each varieties.

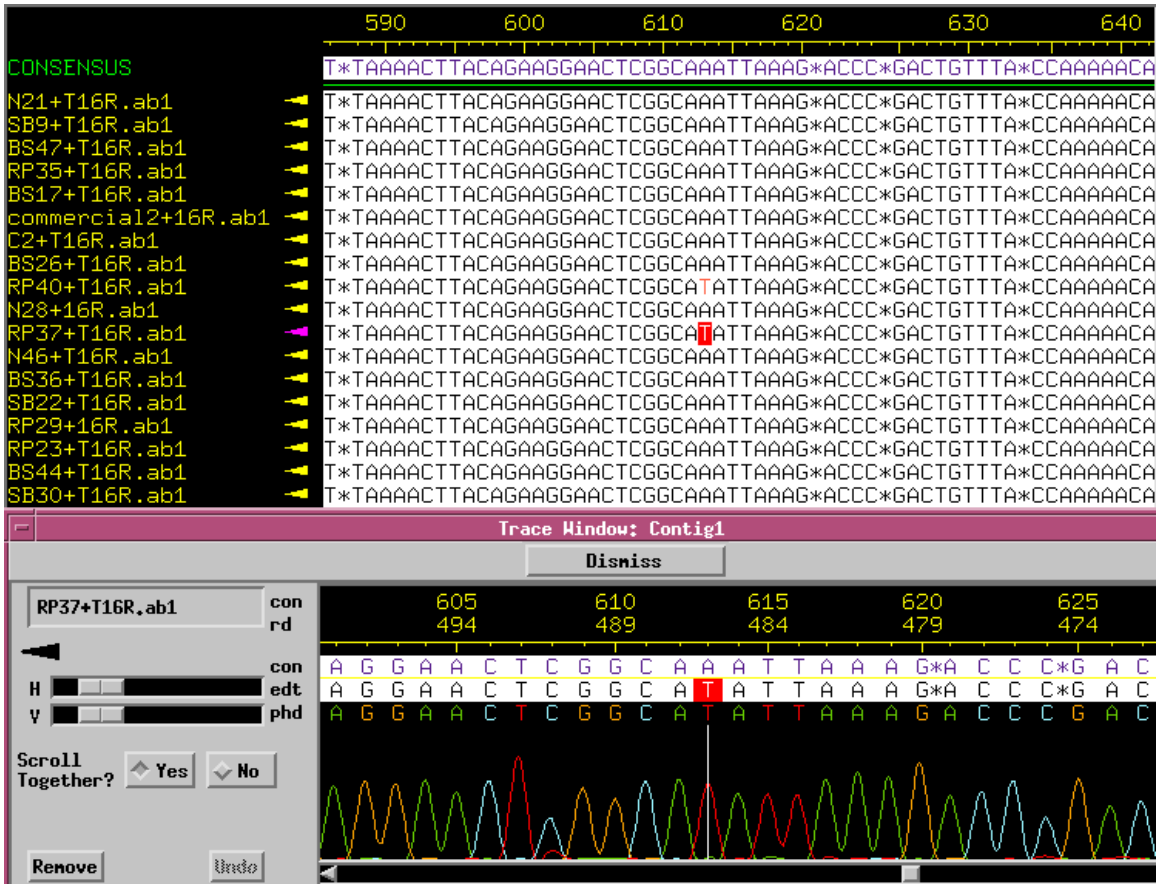


Figure 3.1. Consed alignment showing segregation of a turkey 16S rRNA-based A-T SNP in different turkey varieties

N, RP, C, BS and SB stand for Narragansett, Royal Palm, commercial strain British United Turkeys, Blue Slate and Spanish Black respectively.

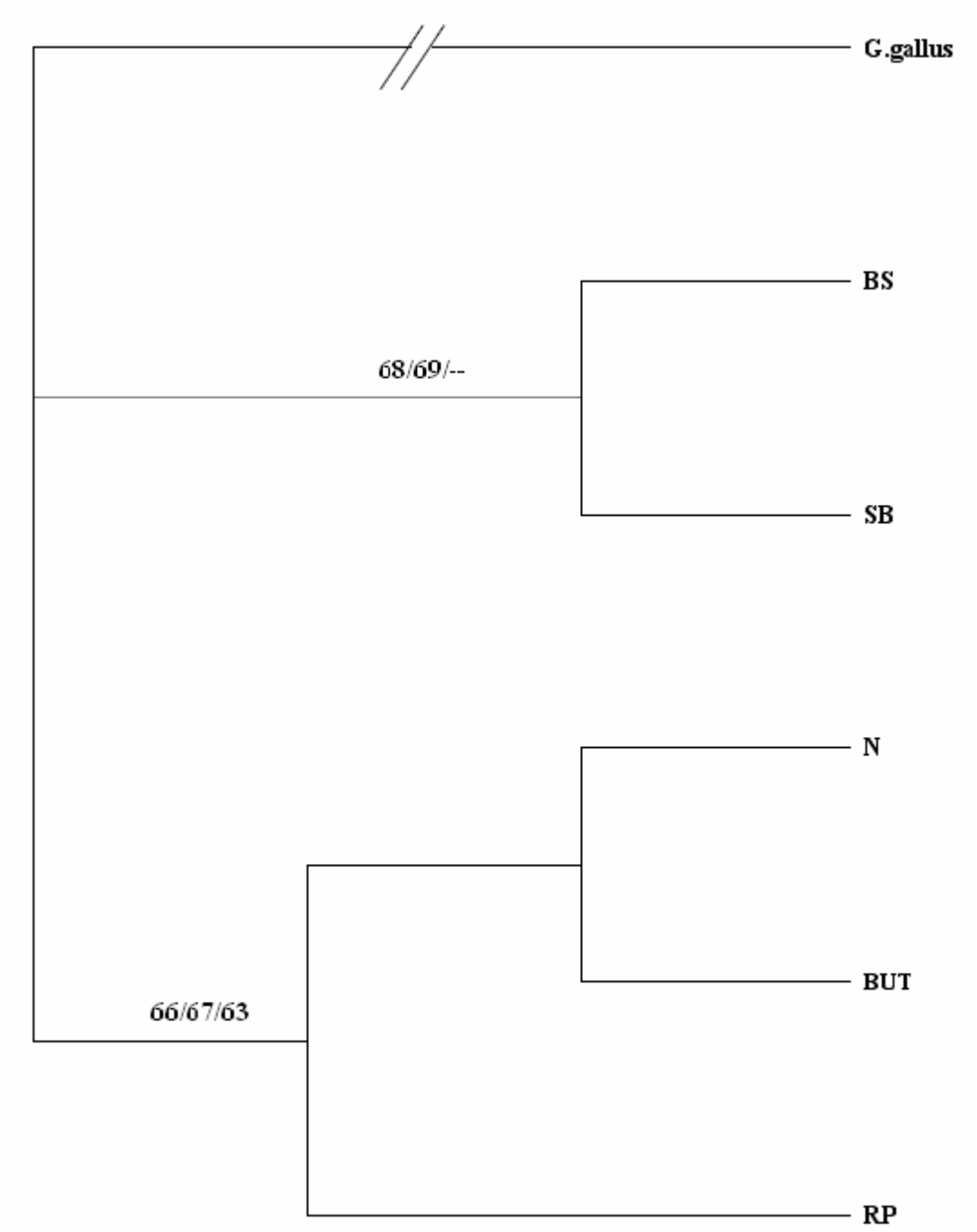


Figure 3.2. D-loop based neighbor joining tree of five turkey varieties and a commercial bird. N, RP, BS and SB represent Narragansett, Royal Palm, Blue Slate and Spanish Black heritage varieties, respectively, and BUT represents a commercial bird from British United Turkeys. The

tree, constructed using the neighbor joining algorithm, was congruent with those from maximum likelihood, minimum evolution and maximum parsimony methods. Confidence of the groupings was estimated using 1000 bootstrap replications. The Arabic numerals at the base of a node are the bootstrap values derived from the maximum parsimony, neighbor-joining, and minimum-evolution analysis, respectively. Bootstrap values lower than 50% are not shown.

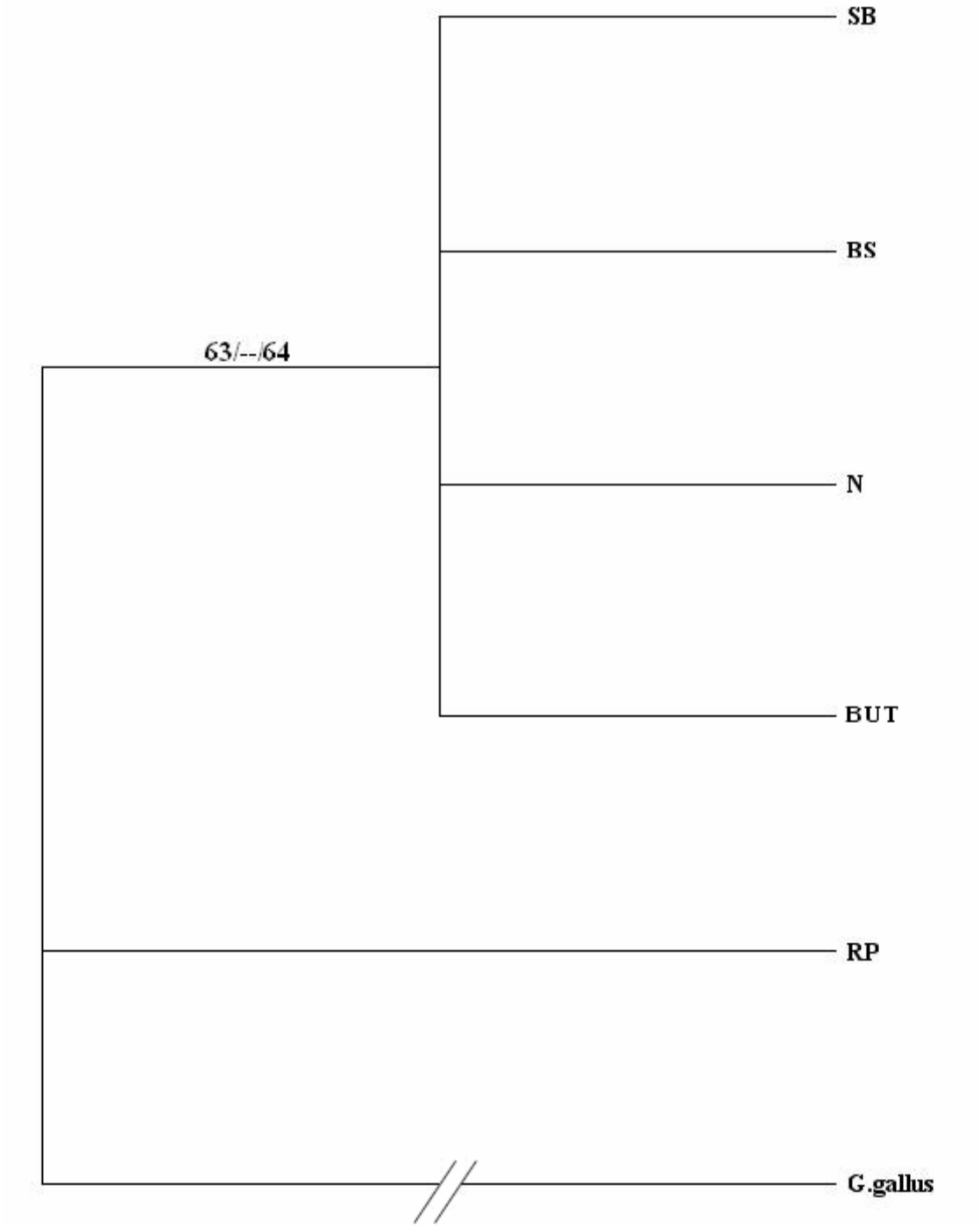


Figure 3.3. 16S rRNA based neighbor joining tree of five turkey varieties and a commercial bird.

N, RP, BS and SB represent Narragansett, Royal Palm, Blue Slate and Spanish Black heritage varieties, respectively, and BUT represents a commercial bird from British United Turkeys. The neighbor joining tree was congruent with those from maximum likelihood, minimum evolution and maximum parsimony methods. Confidence of the groupings was estimated using 1000 bootstrap replications. The Arabic numeral at the base of a node is the bootstrap value. The Arabic numerals at the base of a node are the bootstrap values derived from the maximum parsimony, neighbor-joining, and minimum-evolution analysis, respectively. Bootstrap values lower than 50% are not shown.

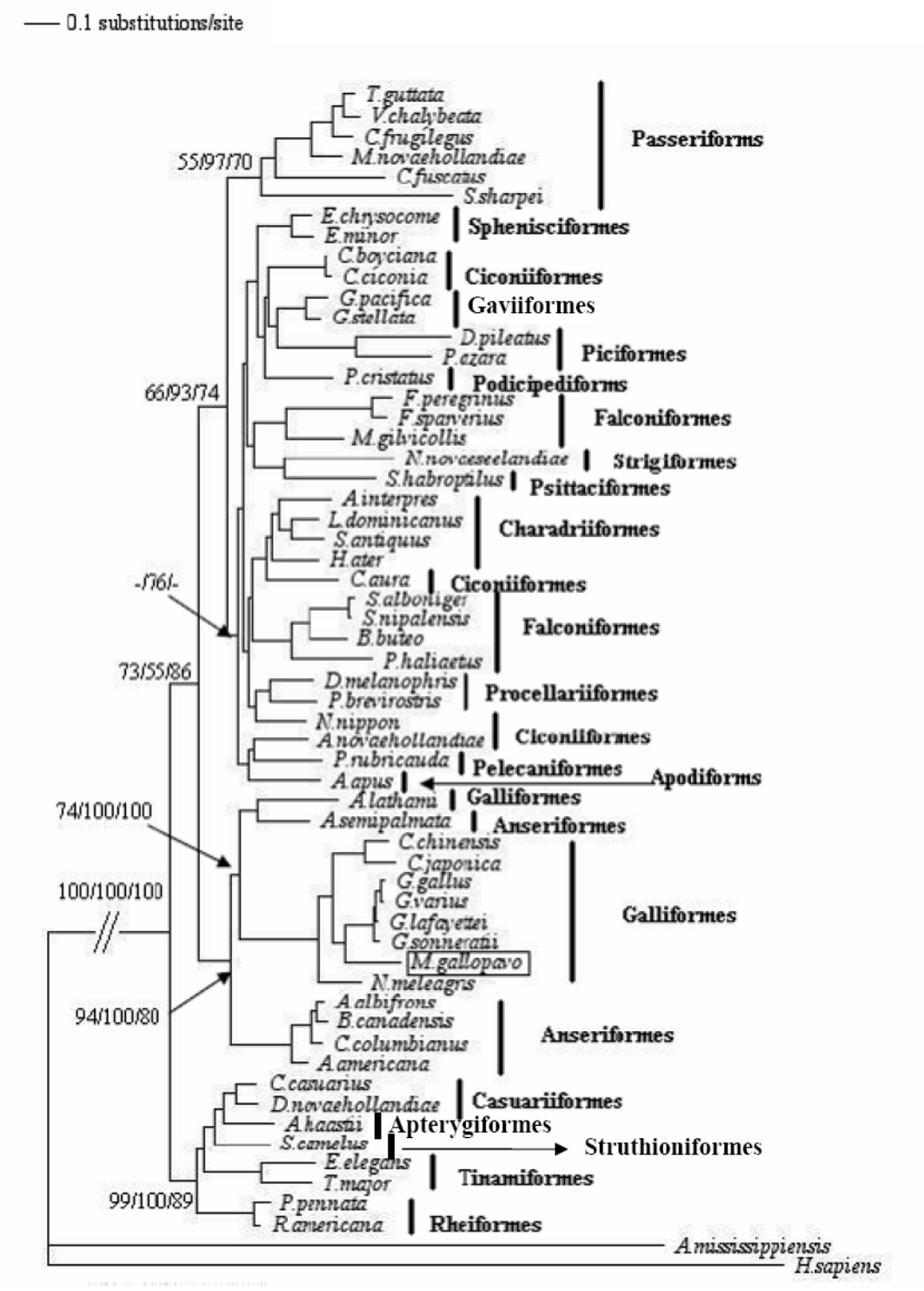


Figure 3.4. A maximum likelihood-based phylogenetic tree constructed using 16S rRNA from 56 avian species described in **Table 4.3**.

The tree was rooted using *A. mississippiensis* (American alligator) and *H. sapiens* (human). The tree was congruent with those from neighbor joining, minimum evolution, and maximum parsimony methods. Confidence of the groupings was estimated using 1000 bootstrap replications. The Arabic numeral at the base of a node is the bootstrap value. The Arabic numerals at the base of a node are the bootstrap values derived from the maximum parsimony, neighbor-joining, and minimum-evolution analysis, respectively. Bootstrap values lower than 50% are not shown.

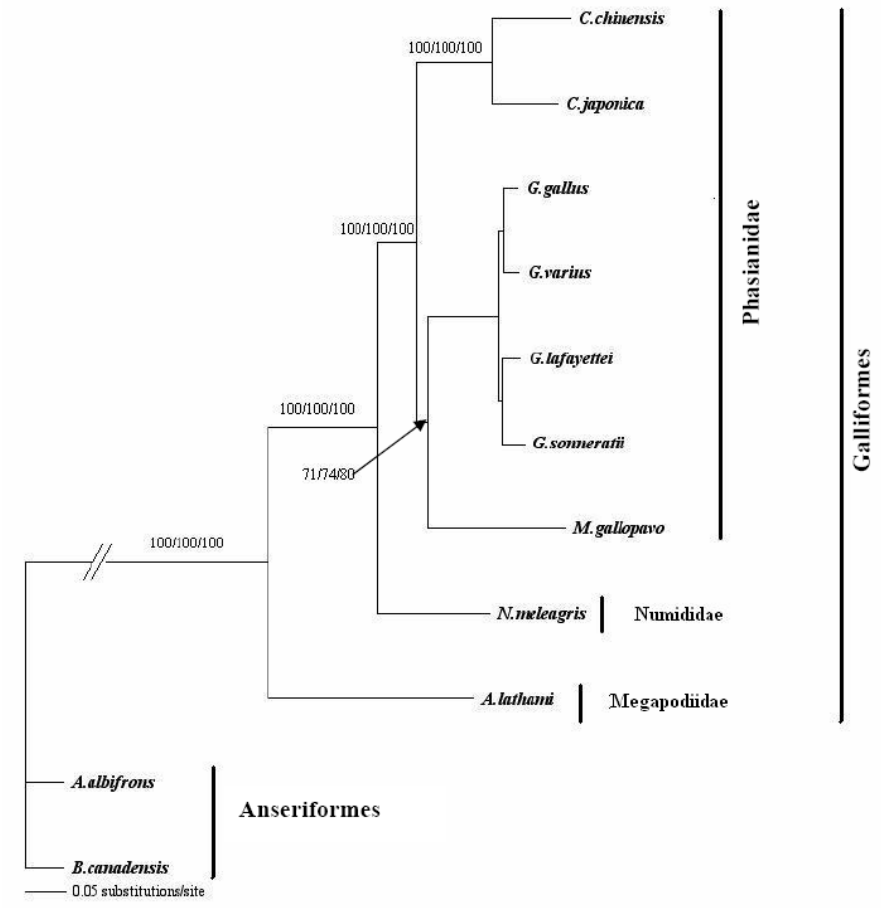


Figure 3.5. A maximum likelihood phylogenetic tree based on the mitochondrial coding region that included 12 protein-coding genes, two rRNA genes, and 19 tRNA genes from Galliformes. The tree was rooted using a mid-point rooting. The tree was congruent with those from neighbor joining, minimum evolution, and maximum parsimony methods. Confidence of the groupings was estimated using 1000 bootstrap replications. The Arabic numeral at the base of a node is the bootstrap value. The Arabic numerals at the base of a node are the bootstrap values derived from the maximum parsimony, neighbor-joining, and minimum-evolution analysis, respectively. Bootstrap values lower than 50% are not shown.

CHAPTER 4

The Mitochondrial Genome Sequence and Molecular Phylogeny of the Budgerigar, *Melopsittacus undulatus*

4.1 Abstract

Budgerigar, *Melopsittacus undulates,* is one of the most widely used birds as pets. Additionally, the budgie is considered a useful model for aging and auditory problems in humans. A limitation on the use of the budgie as a model organism is the lack of knowledge of its genome, both nuclear and mitochondrial. To facilitate the test of the current dissertation hypothesis, the mitochondrial genome (mtGenome) sequence of budgie was developed. We used PCR-based methods described in Chapter 3 to develop a complete budgie mtGenome sequence, and to scan for single nucleotide polymorphisms (SNPs) in the D-loop and 16S rRNA. To evaluate the value of the sequence and provide additional validation of the novel resource, the budgie mtGenome sequence was used to assess the genetic relatedness between the budgie and 57 avian species whose complete mtGenome sequences were publicly available in GenBank. The estimated total length of the mtGenome sequence was 18,193 bp, which included a duplicated hypervariable region, and as expected 13 protein coding genes and 24 RNAs (22 tRNA and 2 rRNA). The duplicated non-coding regions had 86% sequence similarity. The structure of the coding region suggests gene conversion in the budgie mtGenome. Further, the mtGenome sequence-based phylogenetic analysis suggested that the budgerigar was most closely related to kakabo (*Strigops habroptilus*). A total of 22 SNPs was detected that included 20 in one of the control region and two in the 16S rRNA. In addition to their value in phylogenetics and in aging research as done here, these SNPs could facilitate genotype:phynotype assignment. As the first

sequenced Psittaciformes mitochondrial genome, this budgie sequence data could also be a useful resource for understanding specific relationships among these species.

Keyword: budgerigar, mtGenome sequence, phylogenetics

4.2 Introduction

The budgerigar (more commonly known as budgie), *Melopsittacus undulatus*, is a small xerophilous psittacine species that inhabits arid and semiarid grasslands of Australia. Budgies are the most commonly kept pet birds and the most widely used birds for shows and exhibitions around the world (Binks, 1974). In addition, the value of budgie for biomedical research has attracted more and more attention. For example, Psittaciformes, including the budgie, can be used to understand the molecular processes that influence hearing loss and its effect on vocal production since budgie exhibit an assay of vocal plasticity (Farabaugh et al., 1994) and have the ability to regenerate auditory hair cells (Dooling et al., 1997). Budgie is also a useful model for studies of aging because it has exceptional longevity, high relative oxygen consumption, high glucose level and an above average body temperature (Austad, 1998). All these characteristics are considered to be index of higher metabolic rates and oxidative stress level, which decrease the maximum longevity. However, budgies are able to live up to 20 years old, and it is five times that of mammals with comparable body mass, including mice (Holmes and Ottinger, 2003). It is suggested that the budgie may have an innate ability to resist the damage caused by oxidative stress. Therefore, budgie could be useful to understand the genetic basis of tolerance or susceptibility to oxidative stress.

As the "powerhouse of the cell", mitochondria produce most of the reactive oxygen species during energy production, which results in the damage of cell and aging. Because budgie may have some unusual ability to encounter oxidative stress, the mitochondrial genome (mtGenome), which encodes part of the electron transport chain, may have some special characteristics. The importance of the mitochondria in diverse physiological processes makes mutations in the mitochondrial DNA (mtDNA) an important factor in the incidence and severity

of diverse diseases and abnormalities in vertebrates (Linnane et al., 1989; Troen, 2003). For example, mutations especially some single nucleotide polymorphisms (SNPs) in mtDNA have been shown to be associated with diseases like Alzheimer's and Parkinson's (Troen, 2003), diabetes (Maassen et al., 2004), non-hereditary tumors (Zanssen and Schon, 2005), and skeletal and cardiac myopathies (Zeviani and Donato, 2004). For example, a common C-T variant at nucleotide position 16,189 of human mtGenome that was reported by Khogali et al. (2001) to be associated with susceptibility to dilated cardiomyopathy.

Previous study of the mtGenome of *Amazona* parrots, another Psittaciformes species, showed a duplication and rearrangement in the control region. This rearrangement resulted in a new gene order between cytochrome *b* to $tRNA^{Phe}$ (Eberhard et al., 2001). However, this study only sequenced the region from cytochrome *b* to 12S rRNA but not the whole genome. Another Psittaciformes having mtGenome is *Strigops habroptilus*. Unfortunately, this study used clone methods the do the sequencing, and this resulted in the lack of control region (Harrison et al., 2004). So till now, there is no complete mtGenome for Psittaciformes birds.

The evolution of Psittaciformes has also attracted attention in many research groups. There are several studies used nuclear and mtGenome to conduct phylogenetic analysis within parakeets and between parakeets and other species. For example, Russello and Amato (2004) used six partitions including mitochondrial (cytochrome oxidase subunit I, 12S, and 16S) and nuclear (b-fibint7, RP40, and TROP) regions to study the evolutionary history of *Amazona* parrots. The analysis suggested a Neotropical short-tailed parrot genus as sister to *Amazona*. Furthermore, at a finer level, the phylogeny resolved the Greater Antillean endemic species, including the Central American *Amazona albifrons*. Astuti et al. (2006) used cytochrome *b* gene on mtGenome to study the phylogenetic relationship within 27 parrot species at subfamily level,

including Cacatuidae, Loriidae and Psittacidae. The results revealed that Cacatuidae and Loriidae formed monophyletic sister group to other parrots, and Psittacidae formed the paraphyletic sister group. Similarly, Eberhard and Bermingham (2006) mtDNA sequence data (ATPase subunit 6 and 8, cytochrome oxidase subunit I, and cytochrome *b*) to reconstruct the species-level phylogenies for two genera, *Pionopsitta* and *Pteroglossus*. By comparing their results with previous morphology-based phylogenetic analyses, and estimating the absolute timing of lineage and biogeographic divergences, the results suggested that both the *Pionopsitta* and *Pteroglossus* supported a hypothesis of area relationships for high-vagility species, and the time estimates indicated that both genera began to diversify before the start of the Pleistocene.

Surprisingly, the phylogenetic research on budgie is limited. Hedges et al. (1995) used budgie as one avian species to study phylogenetic relationships of the hoatzin. This research used the 12S and 16S rRNA sequences on mtGenome, and the nuclear gene that codes for the eye lens protein, αA-crystallin. The results showed that the hoatzin was most closely related to the typical cuckoos but further away with budgie. Tavares et al. (2006) used both nuclear genes RAG-1, and mitochondrial genes cytochrome *b*, NADH dehydrogenase subunit 2, ATPase subunit 6 and 8, cytochrome oxidase subunit III, 12S rRNA, and 16S rRNA to investigate the phylogenetic relationships in 29 Neotropical parrots including budgie. Both the analysis of nuclear and mitochondrial genes indicated that parrotlet, amazons, macaws and conures grouped together but not with budgie. Kamara (MS thesis) has tested the usage of microsatellite markers to study unrelated budgies which showed limited sequence similarity to both turkey and chicken. The SNP analysis of parrots is also deficient. Shi et al. (2004) used heterologous primers specific for chicken and turkey DNA fragments to generate SNP in the African grey parrot, *Psittacus erythacus*. In that study, seven SNPs were detect on a total of 2.2 kb nuclear genome fragments

and they were confirmed by Mendelian segregation. The lack of the study on budgie might be due to the deficiency of budgie sequence information. The complete mtGenome sequence could be a useful resource of the phylogenetic research.

In the current study, the complete nucleotide sequence of budgie mtGenome is determined, and SNPs in the non-coding region and 16S rRNA are detected. For sequence comparisons, we used the previously published complete mtGenome of 57 other bird species, along with *Alligator mississippiensis* and *Homo sapiens* as outgroup, to investigate the phylogenetic positions of the budgie within the avian tree. The avian species sharing the same rearrangement within their mtGenomes were also used to compare for the gene conversion occurrence.

4.3 Materials and methods

Mitochondrial genome sequencing

Briefly, feather pulp was used as a source of mtDNA as described previously in Chapter 3. Pulp was collected from secondary feathers of birds obtained from a pet store and maintained at VT as described in Chapter 5. It was used to isolate genomic DNA using a minor modification of the recommended protocol for the DNeasy Tissue Kit (Qiagen, Valencia, CA). The modification included the addition of 100 mg/ml dithiothreitol solution to help lysis of the feather pulp during incubation.

The budgie mtGenome sequence was developed from sequencing of PCR products or amplicons obtained using heterologous primers. The primers (**Table 4.1**) included universal oligos previously described by Sorenson et al. (1999) and those developed for the present work (VT Primers). In addition to the conventional criteria for selecting primers, oligos were designed and chosen by Primer 3 (Rozen and Skaletsky, 1997) for their ability to produce overlapping amplicons of 2 to 4 kb. Primers were optimized at an annealing temperature of 56^{o}C using the FailSafe^{TM} PCR PreMixes kit according to the manufacturer's recommendation (Epicentre Technology, Madison, WI). Following optimization, the successful premix that produced a single amplicon was used to carry out PCR for the specific primer pair. For each primer-pair, at least two independent PCR products were purified and sequenced using both reverse and forward primers as previously described (Lin et al., 2006). Internal primers were also developed to complete the sequencing of some long-range PCR products. The internal primers also ensured that some regions of the budgie mtGenome were sequenced at least three times including those regions were two or more primers produced overlapping amplicons. The sequences were

assembled using a combination of bioinformatics tools including of Phred, Phrap and Consed (Gordon et al., 1998) as well as by ClustalW (Higgins et al., 1994).

Validation and annotation

The sequence was validated at two levels: multiple sequencing of each fragment and sequence comparison with GenBank mtGenome sequences of other birds. An additional validation of the budgie mitochondrial DNA sequence was based on sequence similarity analysis of products from multiple birds by ClustalW (Higgins et al., 1994). Further comparison with *Strigops habroptilus* sequence in GenBank (accession number: NC_005931) was as previously described in Chpater 3. To annotate the sequence, BLAST2 (Tatusova & Madden., 1999) and GeneDoc (Nicholas et al., 1997) were used to compare the assembled sequence to database mtGenome sequences. Additionally, ORF-Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and vsfold4 (http://www.rna.itchiba.ac.jp/~vsfold/vsfold4/), a DNA sequence viewer and annotation tool and RNA secondary structure prediction program, respectively, were also used to further annotate and/or validate the BLAST2 and GeneDoc annotation of the budgie mitochondrial DNA sequence.

Phylogenetic analysis

A total of 57 complete mtGenome sequences, each representing a unique avian species, together with the budgie mtGenome described here were used for the relatedness study in different avian species. The 16S rRNA was first chosen to conduct phylogenetic trees for all 57 the birds with two outgroup species: American alligator and human. The multiple sequence alignment was obtained by using ClustalX (Thompson et al., 1997). Phylogenetic analysis was

conducted by PAUP* version 4.0 (Swofford, 2002). Phylogenetic trees were constructed using minimum evolution, neighbor-joining, maximum parsimony, and maximum likelihood methods. One thousand bootstrap replicates were used to assess the confidence in the grouping (Felsenstein and Kishino, 1993). To select an appropriate model for maximum likelihood analysis, we used Modeltest 3.8 (Posada, 2006). According to the Alaike Information Critertion for the 16S rRNA phylogenetic analysis, the General Time Reversible + Gamma + Proportion Invariant (GTR+G+I) model of evolution were selected with gamma distribution shape parameter of 0.5886.

SNP analysis

A PCR-based resequencing method (Smith et al., 2001) was used to search for SNPs in the budgie mtGenome. A total of 20 birds from a commercial pet store (C & P Discount Pet, Virginia) were used to search for sequence variants within the D-loop and 16S rRNA. The newly developed budgie mitochondrial DNA sequence was used to design primers using the web based program Primer 3 (Rozen & Skaletsky, 1997). The primers developed and used were BDLF: 5'-AAAGCCACCCCATTAAAATC-3', BDLR: 5'-AAGGGTGTGTGGGTGTTTTC-3' from the D-loop, and B16SF: 5'-CCTAAAGCAGCCACCCATAA-3', and B16SR: 5'- TAGAAACCGA CCTGGATTGC-3', from the 16S rRNA. The FailSafe^{TM} PCR PreMixes (Epicentre, Madison, WI) were used to optimize the reaction conditions at primer annealing temperature of 56^{o}C. Following the optimization, PCR was carried out, the amplicons purified and sequenced as previously described (Lin et at., 2006). The sequences were analyzed for SNPs using Phred, Phrap and Consed according to Gordon et al. (1998).

4.4 Results and discussion

Seventeen primer pairs amplified products that were sequenced to obtain the budgie mtGenome sequence (**Table 4.1**). All but four primer pairs represent new oligos that could be useful in future investigates involving the budgie mtGenome. A total sequence length of 18,193 bp, representing the budgie mtGenome was obtained from the amplicons produced by these primers. There were totally 13 genes and 24 RNA (22 tRNA and 2 rRNA) identified from annotation of the sequence (**Table 4.2**). Among the 13 protein genes annotated in the budgie mtGenome, only the gene sequence of NADH dehydrogenase subunit 6 (ND6) was on the L-strand. On the other hand, though both of the two rRNA (12S and 16S) were on the H-strand, 9 tRNAs were on the L-strand. Based on the annotation and ClustalW results, the coding region of budgie mtGenome sequence appears to be consistent with other mitochondrial genomic DNA sequences.

Most interesting thing found in budgie mtGenome is the duplication of the control region (**Figure 4.1**). The first control region (CR1) is 1,307 bp in length, and the second one (CR2) is 1,378. By comparing the gene order among non-avian vertebrates and normal avian species, we can predict that CR1 is inserted between $tRNA^{Thr}$ and $tRNA^{Pro}$ (**Figure 4.1**). The similarity of these two control regions is 86%, and about one kb of the sequences of these two control regions were nearly identical, differing only in the 5' and 3' ends. The high similarity indicated that the occurrence of CR1 was caused by the duplication. Because of this duplication, budgie possesses one of the longest mtGenome in avian species. There are totally four avian species having a mtGenome longer than 18,000 bp, and they are black-browed albatross (*Diomedea melanophris*), peregrine falcon (*Falco peregrinus*), ivory billed aracari (*Pteroglossus azara flavirostris*) and

common buzzard (*Buteo buteo*) in GenBank genome database. All these birds have two control regions, and the longest one *Diomedea melanophris* (18,967 bp) even has two ND6.

The most abundant nucleotide in budgie mtGenome sequence of the H-strand was C, followed by A, T and G. This is exactly the same situation in the chicken and human, the frequent is C>A>T>G. While in the mouse, though the A nucleotide is the most frequent as in the turkey, it is followed by T, C and G. The budgie mtGenome has a GC content of only 44.7%. The range of GC content in avian species is 42% to 48% for *Apteryx haastii* and *Aythya americana* respectively according to the current GenBank data. For vertebrate, the lowest is mouse (36%), and highest end is the avian specie. The budgie mtGenome GC content falls in the normal range of vertebrate and avian, and it can be considered to be low when compared with other avian species, such as chicken (46%) and goose (47%). Although it is higher than kakabo (*Strigops habroptilus*, 43%), as the kakabo sequence lack the control region (Harrison et al., 2004), it might be not the truth for kakabo.

An additional nucleotide, missing in the chicken reference sequence (GenBank accession number NC_001323), was observed in NADH dehydrogenase subunit 3 (ND3) at position 174. This additional nucleotide has been reported in 46 birds (Mindell et al., 1998). It has been suggested that the additional nucleotide causes reading frame change, yielding multiple stop codons in ND3 which result in truncated product, though the gene remains functional with this extra base. Mindell et al. (1998) hypothesized that there might be some unknown mechanism to correct this anomalous sequence error such as translational frameshifting and RNA editing.

Fifty-seven whole mtGenome sequences available in GenBank were used to determine the relatedness of the budgie to other birds (**Table 4.3**). The between species 16S rRNA-based minimum evolution, maximum likelihood, maximum parsimony, and neighbor-joining trees

showed a closer relationship between the budgie and kakabo with high bootstrap values which provided strong support for the relationship (**Figure 4.2**). This result is consistent with published taxonomy since both of them are Psittaciformes (Sibley and Ahlquist, 1990).

A total of two SNPs in the 16S rRNA were detected, one C-T transition at nucleotide positions 2,218 and a deletion at 1,822 (**Figure 4.3**). The transition was found in 6 out of 20 budgies, while the deletion was detected in 12 birds. In CR2, there were totally 20 SNPs, and all of them were C-T or A-G transitions. A total of five haplotypes were derived based on the SNPs identified in CR2 with haplotypes 1, 2, 3 had much higher distribution frequencies (**Table 4.4**). The SNPs observed here may provide some resources to investigate genotype:phynotype association analysis in budgie.

In the current study, we have described the budgie mtGenome sequence and SNPs detected in this newly developed genome, and showed an unusual rearrangement of the gene order and control region duplication. Because of the functional importance of mitochondria, the animal mtGenome is generally considered to be under selection for both small size and a conserved gene order (Rand & Harrison, 1986; Quinn & Wilson, 1993; Boore, 1999). Animal mitochondrial genomes rarely contain either introns or intergenic spacers besides the control region: D-loop (Quinn and Wilson, 1993; McKnight and Shaffer, 1997). While the mtGenome gene content and gene order is remarkably stable across vertebrate species, the avian species are an exception to this stability. Several gene order rearrangements have occurred in avian mtGenomes, including the likely ancestral avian gene order first found in the chicken (Desjardins and Morals, 1990). These arrangements primarily affect the area around the ND6 gene, and the avian species have several unusual features in their mitochondrial DNA, such as the lack of the traditional origin of replication for the light strand and the as-yet unidentified splicing function to

repair the insertion found in most avian mtGenomes. For these reasons it is particularly important in avian species to sequence the entire mtGenome, not just individual genes. Especially for Psittaciformes, there is no complete mtGenome sequence. Although *Strigops habroptilus* (Kakabo, NC_005931) is listed in the GenBank, the control region has not been sequenced out (Harrison et al., 2004). Therefore, the budgie mtGenome reported here is very important for both the evolution and phylogenetic studies, and gene function assignment.

Species with the rearrangements typically have a short non-coding region between $tRNA^{Glu}$ and $tRNA^{Phe}$. For example, the forest falcon (*Micrastur gilvicollis*), kestrel (*Falco sparverius*), and woodpecker (*Dryocopus pileatus*) all have the alternative gene order where the control region lies between $tRNA^{Thr}$ and $tRNA^{Pro}$, and a second, unalignable, and often shorter noncoding region lies between $tRNA^{Glu}$ and $tRNA^{Phe}$ (Mindell et al., 1998b). The noncoding regions in the forest falcon and kestrel, much like in the peregrine falcon (Mindell et al., 1998), are mostly repeats of a short sequence: a 4-bp repeat in the forest falcon and a 9-bp sequence in the kestrel. The short woodpecker noncoding region has neither discernable repeats nor any similarity to the woodpecker control region.

However, as previously reported, genus Amazona has two duplicated control region between $tRNA^{Glu}$ and $tRNA^{Phe}$ (Eberhard et al., 2001). This is different to the gene order found in the falcon, as the two control regions are clearly duplicates and can be easily aligned to each other. For instance, the similarity of the two control region of *Amazona ochrocephala auropalliata* has been reported to be about 83% (Eberhard et al., 2001). This duplicated control region has also been observed in other species including osprey (*Pandion haliaetus*) and aracari (*Pteroglossus azara flavirostris*, Gibb et al., 2007), both of which had the duplicated regions located between $tRNA^{Thr}$ and $tRNA^{Pro}$, and $tRNA^{Glu}$ and $tRNA^{Phe}$, respectively (Gibb et al.,

2007). In all these species, the two control regions are nearly identical, differing only in the 5' and 3' ends. All plausible avian phylogenies implied that these rearrangements arose independently several times in birds (Mindell et al., 1998b). There are several mechanisms that may result in gene rearrangements in a circular genome, such as recombination, slipped-strand mispairing, errors in synchronizing the points of initiation and termination, and illicit priming of replication by tRNAs near the replication origin (Mindell et al., 1998b; Mueller and Boore, 2005; Gibb et al., 2007). It is possible that any combination of these mechanisms could be responsible for the gene orders seen in these birds (Mueller and Boore, 2005).

The budgie mtGenome sequence presented here provides insight into the evolutionary relationships of Psittaciformes. Many studies suggest that gene order has the potential for being an excellent marker for determining phylogenetic relationships (Snel et al., 2005; Steel and Penny, 2005; Boore, 2006). In the current chapter, phylogenetic analysis using 16S rRNA showed that the budgie is most closely related to the kakabo, an observation that is consistent with the DNA-DNA hybridization analysis of Sibley and Ahlquist (1990). However, as shown in **Figure 3.4**, kakabo appears to be closest to morepork (*Ninox novaeseelandiae*) if the budgie data is not included. Consistent with our results, Gibb et al. (2007) observed that by using 12 protein-coding genes, 2 rRNAs and 21 tRNAs (lacking $tRNA^{Phe}$ and ND6), kakabo was most closely related to morepork. The budgie mtGenome sequence appears to have resolved the question of Strigiformes and Psittaciformes relatedness. The Psittaciformes including the budgie and kakabo appear to be in the same branch with 100% bootstrap value, and they are grouped together with Passeriformes and Strigiformes like morepork. There were also other differences between the current phylogenetic tree and **Figure 3.4**, such as the the grouping of Falconifomes. However, because the branch lengths of the different goupings were not long in both of the trees, the

changes were not revolutionary when adding the budgie except the relatedness among Psittaciformes, Passeriformes and Strigiformes.

In summary, the budgie mtGenome sequence was developed and annotated. Before using it to test the hypothesis of this dissertation research, we used the sequence to evaluate the genetic relatedness between the budgie and other birds, especially Passeriformes and Strigiformes, a relationship that do not have a consensus. The mitochondrial sequence also represents the first for a Psittaciformes. The rearrangement of gene order based on duplication of the control region appears to be consistent with that seen in *Amazona* parrots that previously described (Eberhard et al., 2001). The budgie mtGenome sequence provides a resource for phylogenetic and population genetic analyses because the evolutionary relationships among avian species continues to be without a general consensus. The whole mtGenome sequence described here will be useful for generating more data needed to understand the evolutionary relationships of the budgie as well as to understand the role and origin of the rearrangement of the control region. Since it is known that mutations in the mitochondria are sometimes associated with diseases and abnormalities in human and model organisms (Wallace, 2005), the sequence and the SNPs described provide a foundation for determining the role of the budgie mitochondria in diseases and its extraordinary ability to deal with oxidative stress (Austad, 1997).

Table 4.1. Primers designed and developed for PCR-based budgie sequencing.

Primer ID	Primer sequence
BL1F[a]	AARCCMGAATGRTAYTTYCTWTTYGC
BL1Rnew	AAGGGTGTGTGGGTGTTTTC
BL1in1F	TTCTGGGCATTGCATAATTG
BL1in2F	TGAAGGCTTATCCCTTCTTAGAG
BL1in2R	GGTTGTAGGTGGGGAGGAGT
BL2F[a]	YAAAGCATGRCACTGAA
BL2R[a]	TYTCAGGYGTARGCTGARTGCTT
BL3Fnew	ATACCCCCAACGAAAAGAGG
BL3Rnew	GGCCTAGTTCTGCTCGAATG
BL3in1F	CAGCACCACACAGCAAAGAT
BL3in1R	CTGGGTTGCATTCAGGAGAT
BL3in2F	CACACCCTGTTAACCCAACC
BL3in2R	GGATGATGAGCCACTTTGGT
BL3in3F	GAGTTCAGACCGGAGCAATC
BL3in3R	GGTGGGTGCGAGCTTTATTA
BL4F[a]	CCYCTGTAAAAAGGWCTACAGCC
BL4R[a]	GGRTCRAANCCRCAYTCRTANGG
BL4in1F	CGGAGCAATCAACTTCATCA
BL4in1R	CGCCTGAGGCTAGTAGGATG
BL4in2F	ACATCGCCCTACATGACACA
BL4in2R	TTGGTGGGCCATTAAATGTT
BL4in3F	CCTCTACATGGCCAACCACT
BL4in3R	AGGTGTCCTAGGGAGGCTGT
BL5F[a]	CAYCAYTTYGGNTTYGAAGCHGC
BL5R[a]	TGNAGDGCDGCDGTRTTDGC
BL5in1F	AGGCCTAGAATGGGCAGAAT
BL5in1R	GATGGTTTTGGGGGAGTTTT
BL5in2F	ACAACACGAACCCCTCTCAC
BL5in2R	TGGCTTTAGGATCAGGAGGA
BL6F[a]	ATCCRTTGGTCTTAGGARCCA
BL6R[a]	CTTCANTYTTTGGYTTACAAGRCC
BL6in1F	CTCATCCTAGCCGCTACAGG
BL6in1R	AGCTAGGCCTGCGATTATGA

[a] Universal primers described by (Sorenson et al., 1999) were also used.

Table 4.2. Sequence annotation of the mitochondrial genome of the budgie, *Melopsittacus undulatus.*

Genes	Location	Size (bp)	Codon/ initial	Codon/ terminal
tRNA-Phe	1-67	67		
12S ribosomal RNA (12S rRNA)	68-1044	977		
tRNA-Val	1045-1116	72		
16S ribosomal RNA (16S rRNA)	1117-2688	1572		
tRNA-Leu	2689-2763	75		
NADH dehydrogenase subunit 1 (*ND1*)	2770-3748	979	ATG	TAA
tRNA-Ile	3749-3820	72		
tRNA-Gln	3825-3895[a]	71		
tRNA-Met	3895-3963	69		
NADH dehydrogenase subunit 2 (*ND2*)	3964-5004	1040	ATG	TAA
tRNA-Trp	5004-5074	71		
tRNA-Ala	5076-5144[a]	69		
tRNA-Asn	5147-5221[a]	75		
tRNA-Cys	5228-5294[a]	67		
tRNA-Tyr	5294-5364[a]	71		
Cytochrome oxidase subunit 1 (*COX1*)	5375-6922	1548	GTG	AGG
tRNA-Ser	6914-6989[a]	76		
tRNA-Asp	6993-7061	69		
Cytochrome oxidase subunit 2 (*COX2*)	7064-7747	684	ATG	TAA
tRNA-Lys	7749-7818	70		
ATPase subunit 8 (*ATPase8*)	7820-7987	168	ATG	TAA
ATPase subunit 6 (*ATPase6*)	7978-8661	684	ATG	TAA
Cytochrome oxidase subunit 3 (*COX3*)	8661-9446	786	ATG	TGC
tRNA-Gly	9446-9513	68		
NADH dehydrogenase subunit 3 (*ND3*)	9514-9865	352	ATG	TAA
tRNA-Arg	9865-9934	70		
NADH dehydrogenase subunit 4 light-chain (*ND4L*)	9936-10232	297	ATG	TAA
NADH dehydrogenase subunit 4 (*ND4*)	10226-11620	1395	ATG	TGC
tRNA-His	11619-11687	69		
tRNA-Ser	11689-11752	64		
tRNA-Leu	11753-11823	71		
NADH dehydrogenase subunit 5 (*ND5*)	11824-13638	1815	GTG	TAA
Cytochrome b (*Cytb*)	13650-14789	1140	ATG	TAA
tRNA-Thr	14789-14856	68		
tRNA-Pro	16164-16232[a]	69		
NADH dehydrogenase subunit 6 (*ND6*)	16236-16745[a]	510	ATG	TAG
tRNA-Glu	16748-16815[a]	68		

[a] Coded on the complementary (L) strand.

Table 4.3. Scientific and common names of birds for which whole mitochondrial genome sequences are available in GenBank.

Bird	Accession	Common name
Alectura lathami	NC_007227	Australian brush-turkey
Alligator mississippiensis [b]	NC_001922	American alligator
Anser albifrons	NC_004539	White-fronted goose
Anseranas semipalmata	NC_005933	Magpie goose
Apteryx haastii	NC_002782	Great spotted kiwi
Apus apus	NC_008540	Swift
Ardea novaehollandiae	NC_008551	White-faced heron
Arenaria interpres	NC_003712	Ruddy turnstone
Aythya americana	NC_000877	Redhead
Branta canadensis	NC_007011	Canada goose
Buteo buteo	NC_003128	Common buzzard
Casuarius casuarius	NC_002778	Southern cassowary
Cathartes aura	NC_007628	Turkey vulture
Ciconia boyciana	NC_002196	Oriental stork
Ciconia ciconia	NC_002197	White stork
Cnemotriccus fuscatus	NC_007975	Fuscous flycatcher
Corvus frugilegus	NC_002069	Rook
Coturnix chinensis	NC_004575	Blue-breasted quail
Coturnix japonica	NC_003408	Japanese quail
Cygnus columbianus	NC_007691	Tundra swan
Diomedea melanophris	NC_007172	Black-browed albatross
Dromaius novaehollandiae	NC_002784	Emu
Dryocopus pileatus	NC_008546	Pileated woodpecker
Eudromia elegans	NC_002772	Elegant crested-tinamou
Eudyptes chrysocome	NC_008138	Rockhopper penguin
Eudyptula minor	NC_004538	Little blue penguin
Falco peregrinus	NC_000878	Peregrine falcon
Falco sparverius	NC_008547	American kestrel
Gallus gallus	NC_001323	Chicken
Gallus lafayettei	NC_007239	Ceylon junglefowl
Gallus sonneratii	NC_007240	Gray junglefowl
Gallus varius	NC_007238	Green junglefowl
Gavia pacifica	NC_008139	Pacific loon
Gavia stellata	NC_007007	Red-throated loon

Haematopus ater	NC_003713	Blackish oystercatcher
Homo sapiens [b]	NC_001807	Human
Larus dominicanus	NC_007006	Southern black-backed gull
Menura novaehollandiae	NC_007883	Superb lyrebird
Meleagris gallopavo	EF_153719	Domestic turkey
Melopsittacus undulates [a]	**EF450826**	Budgerigar
Micrastur gilvicollis	NC_008548	Lined forest-falcon
Ninox novaeseelandiae	NC_005932	Morepork
Nipponia nippon	NC_008132	Crested ibis
Numida meleagris	NC_006382	Helmeted guineafowl
Pandion haliaetus	NC_008550	Osprey
Phaethon rubricauda	NC_007979	Red-tailed tropicbird
Podiceps cristatus	NC_008140	Great crested grebe
Pterocnemia pennata	NC_002783	Lesser rhea
Pterodroma brevirostris	NC_007174	Kerguelen petrel
Pteroglossus azara flavirostris	NC_008549	Ivory billed aracari
Rhea americana	NC_000846	Greater rhea
Smithornis sharpei	NC_000879	Grey-headed broadbill
Spizaetus alboniger	NC_007599	Blyth's hawk-eagle
Spizaetus nipalensis	NC_007598	Mountain hawk-eagle
Strigops habroptilus	NC_005931	Kakapo
Struthio camelus	NC_002785	Ostrich
Synthliboramphus antiquus	NC_007978	Ancient murrelet
Taeniopygia guttata	NC_007897	Zebra finch
Tinamus major	NC_002781	Great tinamou
Vidua chalybeata	NC_000880	Steelblue widowfinch

[a] The sequence developed in this work..

[b] Species used as outgroup.

Table 4.4. Budgie Control Region 2 (CR2) haplotypes and diversity.

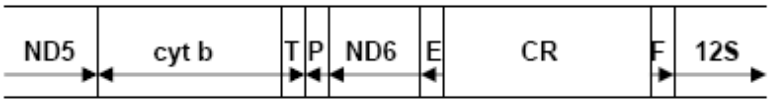
ID	Haplotype[a]	Frequency[b]
BCR2HAP1	-A-A-C-C-A-C-C-T-T-T-A-G-T-T-G-T-A-T-A-T-	0.25
BCR2HAP2	-A-G-C-C-G-C-T-C-T-C-G-G-T-T-A-T-A-T-A-T-	0.292
BCR2HAP3	-G-G-T-T-G-T-T-T-C-C-G-G-T-C-G-T-A-C-A-C-	0.375
BCR2HAP4	-A-G-C-C-G-C-T-C-T-C-G-G-C-T-G-C-G-T-G-T-	0.042
BCR2HAP5	-A-G-C-C-G-C-T-C-T-C-G-A-T-T-G-T-A-T-A-T-	0.042

[a] The SNP position (according to GenBank accession number: EF450826) were listed correspond to each site as follows: 16518, 16694, 16816, 16824, 16831, 16942, 16945, 16972, 16979, 16980, 16996, 16998, 17016, 17032, 17053, 17063, 17106, 17918, 18079, 18112, and 18129, respectively.

[b] Haplotype frequency was determined in a total of 24 budgies.

(a) Standard non-avian vertebrate mitochondrial gene order:

(b) Standard avian mitochondrial gene order:



(c) Budgerigar (*Melopsitaccus undulates*) mitochondrial gene order:

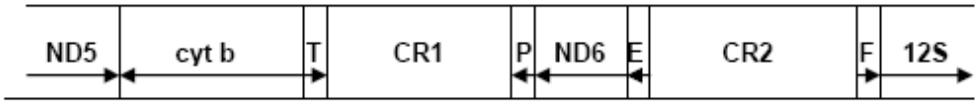


Figure 4.1. Schematic diagrams of the gene order of the area surrounding the control region found in vertebrate mitochondrial genome (not drawn to scale).

Arrows in this figure represent the gene directionality.

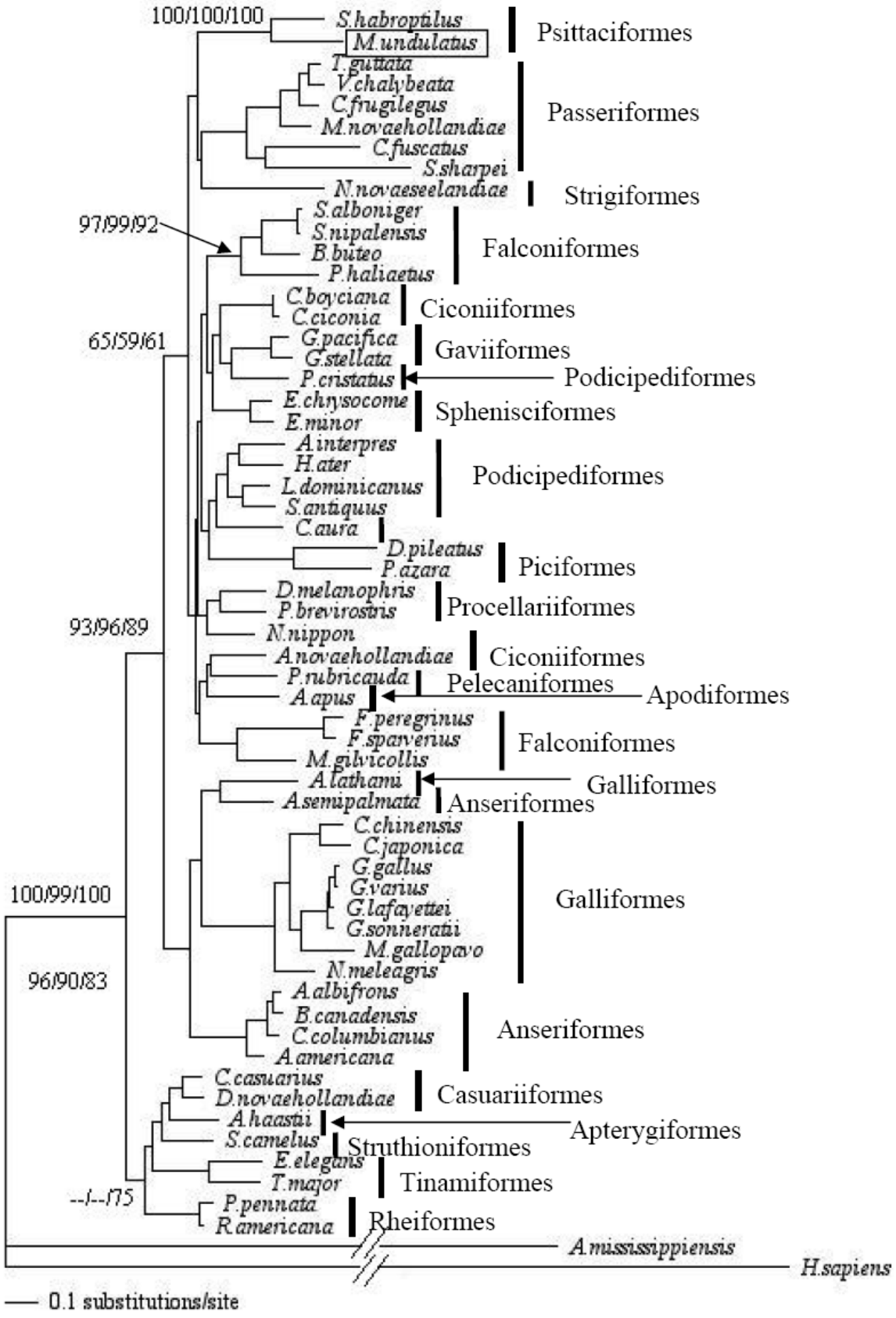

100/100/100
S.habroptilus
M.undulatus
Psittaciformes
T.guttata
V.chalybeata
C.frugilegus
M.novaehollandiae
C.fuscatus
S.sharpei
Passeriformes
N.novaeseelandiae
Strigiformes
97/99/92
S.alboniger
S.nipalensis
B.buteo
P.haliaetus
Falconiformes
C.boyciana
C.ciconia
Ciconiiformes
65/59/61
G.pacifica
G.stellata
Gaviiformes
P.cristatus
Podicipediformes
E.chrysocome
E.minor
Sphenisciformes
A.interpres
H.ater
L.dominicanus
S.antiquus
C.aura
Podicipediformes
D.pileatus
P.azara
Piciformes
D.melanophris
P.brevirostris
Procellariiformes
93/96/89
N.nippon
A.novaehollandiae
Ciconiiformes
P.rubricauda
Pelecaniformes
A.apus
Apodiformes
F.peregrinus
F.sparverius
M.gilvicollis
Falconiformes
A.lathami
Galliformes
A.semipalmata
Anseriformes
C.chinensis
C.japonica
G.gallus
G.varius
G.lafayettei
G.sonneratii
M.gallopavo
N.meleagris
Galliformes
100/99/100
96/90/83
A.albifrons
B.canadensis
C.columbianus
A.americana
Anseriformes
C.casuarius
D.novaehollandiae
Casuariiformes
A.haastii
Apterygiformes
S.camelus
Struthioniformes
E.elegans
T.major
Tinamiformes
--/--/75
P.pennata
R.americana
Rheiformes
A.mississippiensis
H.sapiens
0.1 substitutions/site

Figure 4.2. Phylogenetic tree conducted by using 16S rRNA of the avian species.

All trees were rooted using the *A. mississippiensis* (American alligator) and *H. sapiens* (human). The tree shown here is a phylogram constructed using the maximum likelihood algorithm. Neighbor-joining, minimum evolution and maximum parsimony methods have also been used in the analysis. For all the methods, confidence of the groupings is estimated using 1000 bootstrap replications. The Arabic numeral at the base of a node is the bootstrap value that represents percent of times out of 1000 bootstrap re-samplings that branches were grouped together at a particular node. The numbers is the bootstrap value derived from the neighbor-joining, minimum-evolution and maximum parsimony analysis respectively. Bootstrap values lower than 50% are not shown.

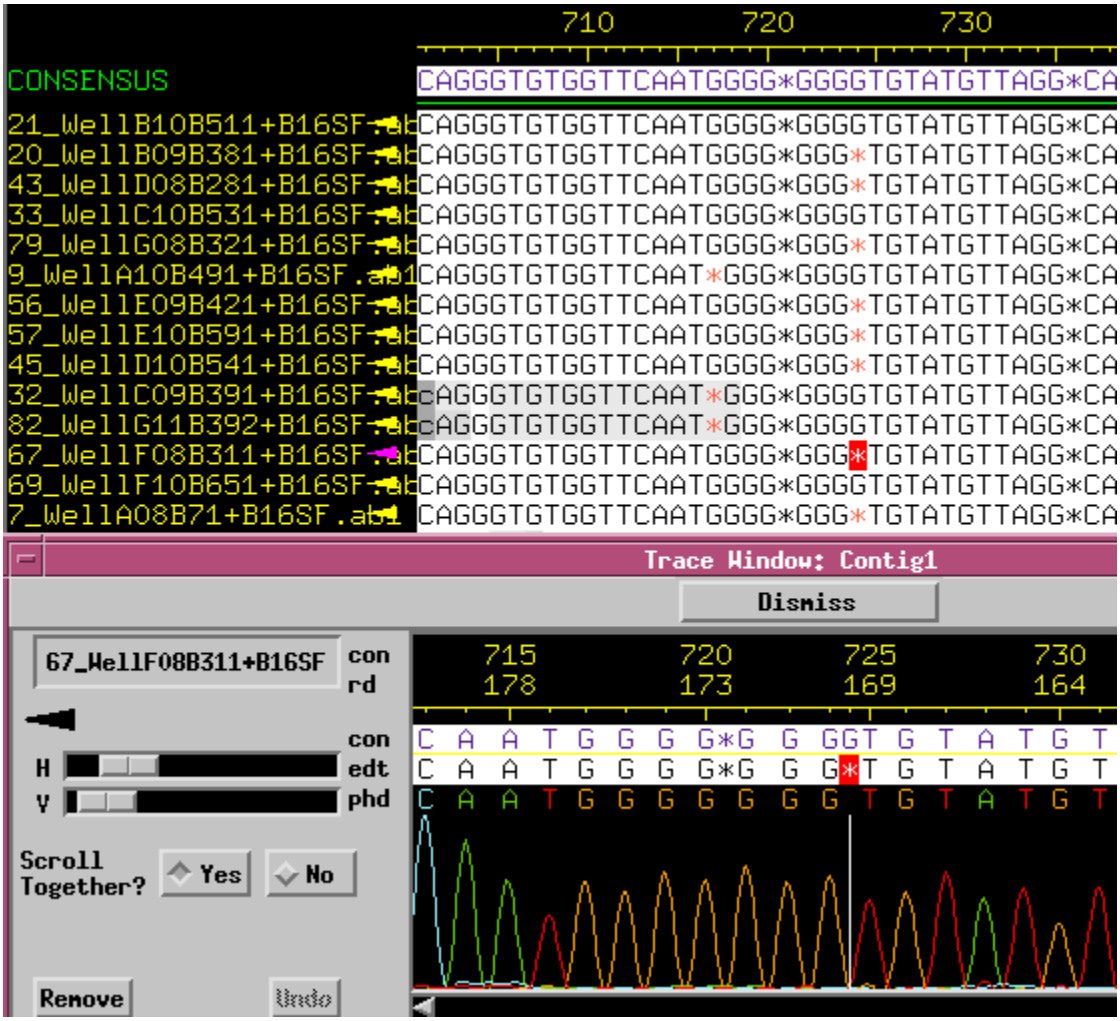


Figure 4.3. Consed alignment showing segregation of budgie 16S rRNA-based deletion at position 1.822 according to the budgie mitochondrial genome (GenBank Accession number: EF450826).

CHAPTER 5

Oxidative Stress, Mitochondrial DNA Mutations, and Longevity in Birds

5.1 Abstract

The mitochondrial theory of aging stipulates that oxidative damage affects mitochondrial genome, causing decreased longevity. To test this theory, we evaluated differences in oxidative stress and mtDNA mutations in four bird species of varying longevities including the quail and budgie with average lifespan of 3 and 18 years, respectively. Using the biomarkers for oxidative stress including thiobarbiturate acid reacting substance (TBARS), and plasma uric acid (PUA) and glutathione (GSH), measurements were taken at 10, 30, 55 and 80 wks-of-age within each species. The PUA level was highest in guineafowl and budgie, but lowest in quail. However, the level of GSH was higher in guineafowl, but lower in budgie. On the other hand, PUA significantly decreased with age (P<0.05), while TBARS, a lipid peroxidation index, increased. Results showed that age, species, body weight, and sex significantly (P<0.05) affected the level of oxidative stress in each of the four species. The total number of SNPs within the D-loop and 16S rRNA in each of the four species ranged from zero in the quail to 22 in the budgie. This number was unaffected by age. In summary, though oxidative stress within each species appeared to increase with age, nucleotide variation in mitochondrial genome did not. It appears that the present work was in consistent with recent reports that the effect or association of oxidative stress on longevity is not mediated through mutations in the mitochondrial DNA.

Keywords: birds, oxidative stress, mitochondrial genome, longevity, single nucleotide polymorphism

5.2 Introduction

Oxidative stress, a result of an imbalance between oxidants and antioxidants, is believed to be responsible for many diseases and abnormalities in eukaryotes (Harman, 1981). Animals have developed a strategy to counter free radicals by producing enzymatic and non-enzymatic antioxidants, including superoxide dismutase, glutathione peroxidase, and catalase, glutathione, uric acid, and vitamins (Miller et al., 1993). These antioxidants can scavenge free radicals by either removing superoxide and peroxides before they react with other catalysts to form more reactive species, or terminating the peroxidative chain reactions initiated by free radicals released from enzymatic degradation (Miller et al., 1993; Schulz et al., 2000).

Oxidative stress has been implicated in organismal aging and it is believed to be the central paradigm in the "Free Radical Theory of Aging" (Harman, 1956). The mtDNA/ oxidative stress hypothesis, derived from the "Free Radical Theory of Aging", represents the relationship between oxidative stress, mtDNA mutation, and aging. It stipulates that reactive oxygen species contribute significantly to the somatic accumulation of mtDNA mutations, leading to the gradual loss of bioenergetic capacity and eventually resulting in aging and cell death (Toren, 2003). Several studies have tried to validate this hypothesis in birds and other vertebrates. For example, Barja and Herrero (2000) evaluated mtDNA damage and longevity of different animals. Using 8-oxo-dG as a biomarker for oxidative DNA damage, they compared short-lived species including the mouse, which lives only 3.5 years, to long-lived species, including the cattle and horse, with an average life span of over 40 years. The comparisons revealed an inverse correlation between 8-oxo-dG/10^5dG in mtDNA and life span.

The damage of mtDNA caused by free radicals probably occur during replication when single stranded DNA is particularly susceptible to oxidative damage (Reyes et al., 1998). The

spectrum of base substitution mutations that accumulate in aged individuals differs across tissues. For instance, Wang et al. (2001) detected different age-dependent accumulations of point mutation A189G and T408A in mtDNA replication control sites in skeletal muscle and skin. It was suggested that this may be due to variations in the mechanism of replication in different tissues. On the other hand, though mitochondria possess base excision repair enzymes that are capable of repairing damaged bases in, the activity of enzymes apparently declines with age (Stuart and Brown, 2006). For example, Imam et al. (2006) studied differential changes in the repair of oxidative DNA damage in various brain regions during aging in mice. The results indicated that there was a significant age-dependent decrease in incision activities of glycosylases in the mitochondria of all brain regions, whereas variable patterns of changes independent of age were seen in nuclei.

Several studies have unambiguously demonstrated that mtDNA base substitution mutations accumulate as a result of aging in a variety of tissues and species, including rodents (Zhang et al., 2003), rhesus (Lopez et al., 2000), monkeys (Kujoth et al., 2007), and humans (Silvestri et al., 1998). For example, the frequency of the A3243G mtDNA mutation, which causes several disorders including maternally inherited mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome, increased with age in the skeletal muscle of normal humans (Zhang et al., 1998). Using a PCR-based method, Lin et al. (2002) determined that the brains of elderly human subjects had a high aggregate of mtDNA base-substitution mutations up to a frequency of 2×10^{-4} mutations/bp.

As a complement to these laboratory models, the budgie and other birds have been proposed as a potential resource for studying specific mechanisms that underlie aging (Austad, 1991). Although the budgie and quail have similar body weight and size, the budgie lives, on

average, five times longer than the quail, even with a higher metabolic rate and lifetime oxygen expenditure especially when compare with the much bigger guineafowl and turkey (Holmes and Austad, 1995). The budgie's lifespan suggests that it has an extraordinary capability to resist the effects of oxidative stress caused by the high metabolic rate. While there have been many investigations of the relationship between oxidative stress and other factors including age in bird, none, to our knowledge, looked at the effect on mtDNA mutations. For example, Pirsljin et al. (2006) recently described changes in the blood level of TBARS and GSH in chicken following dietary restriction and fasting. In an earlier study, Enkvetchakul et al. (1995) reported higher levels of blood GSH in older chickens, quails and turkeys. Similarly, Herrero and Barja (1999) assessed the mtDNA damage in pigeon and budgie by using 8-oxo-dG as biomarker, which indicated that long-lived birds had lower oxidative DNA damage than short-lived mammals including rats and mice.

Here, we hypothesized that oxidative stress was the primary biological mechanism responsible for differences in longevity among birds and that this difference correlated with mitochondrial genomic DNA mutations. We selected the budgie, guineafowl, turkey and quail because of their distinct average life spans, and evaluated them at different ages for oxidative stress and mtDNA mutations.

5.3 Materials and Methods

Animals and sample collection

The primary rationale for the four species used (**Figure 5.1**) was the previous descriptions of age, ease of availability and of rearing. The heritage domestic turkeys (n=28) used were from random mating of birds maintained at the VT Turkey Farm which were originally from *Privett Hatcheries* (New Mexico). The Japanese quails (n=40) were obtained from the Department of Biology, courtesy of Dr. Anne McNabb. Guineafowls (n=18) were obtained from a commercial hatchery (*Fairview Hatchery*, Indiana). Budgies (n=21) were bought from a local pet store at the age under 6 months. All the birds were maintained in artificial conditions and raised according to standard methods and according to the guidelines of Virginia Tech's Institutional Animal Care and Use Committee (**Table 5.1, Figure 5.1**). To reflect conditions in the wild as much as possible which could affect blood levels of biomarkers especially GSH and TBARS (Pirsljin et al., 2006), birds were neither fasted, nor feed-restricted.

Blood was collected for biomarker analysis by brachial venipuncture into tubes containing EDTA as anticoagulant is needed. The blood collection time points for budgie were 30 and 55 wks-of-age, for guineafowl were 3, 10, 30, 55 and 80 wks-of-age, while both quail and turkey blood samples were collected at 10, 30, 55 and 80 wks-of-age. At the time of blood collection, each bird was weighed using a standard scale. Plasma was obtained by centrifuging the blood samples at approximately 10,000x g for 15 minutes at 4°C. Deproteinated whole blood was obtained by centrifuging at 3, 000 x g for 5 minutes with 50 ul of 5% metaphosphoric acid per 50 ul EDTA treated whole blood. All the samples were stored at -20°C until ready for use. While plasma was used for the analyses of TBARS and PUA, deproteinated whole blood was used to determine levels of GSH.

Biomarker Assays

Our choice of multiple biomarkers as predictors was because there was no consensus in the literature and among experts of a single biomarker that was a reliable indicator of oxidative stress level in vertebrates (Fang et al., 2002). Three biomarkers, chosen for the ease of use and the previously use in birds in literatures (Hartman et al., 2006; Enkvetchakul et al., 1995; Pirsljin et al., 2006), were evaluated including plasma uric acid (PUA), glutathione (GSH) and thiobarbituric reactive substances (TBARS).

Plasma Uric Acid (PUA)

Uric acid concentration in blood has become a simple and direct measurement of oxidative stress level without any pretreatment (Yazar et al., 2003). The PUA assay in our lab, especially in the turkey, has previously been described (Hartman et al., 2006). Duplicate plasma samples from each bird were assayed for PUA using an optimized commercial enzymatic assay (*Diazyme,* La Jolla, CA) which was based on the following reactions:

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2H_2O_2 + \text{ESPAS} + \text{4-aminopyrene} \xrightarrow{\text{peroxidase}} \text{Quinine dye} + 4H_2O$$

The quinine dye was monitored by spectrophotometer at a wavelength of 600 nm. Following optimization, the manufacturer's recommended protocol was modified by doubling the reagents in all the PUA assays.

Glutathione (GSH)

The GSH assay used here is a modification of the method first described by Tietze (1969). The general thiol reagent reacts with GSH to form the chromophore. Reduced and

oxidized GSH were measured using a spectrophotometric assay at 412 nm according to modification of the recommended procedure of the assay kit (*Northwest Life Science Specialties,* Vancouver, WA). The modification involved dilution of each sample 40 fold, and dilution of calibrators 5 fold to keep the readings in range of the UV spectrophotometer. Duplicate samples from each bird were assayed. The absorbance was recorded at 60-second intervals for 10 minutes. The GSH concentration of each sample was calculated from the absorbance reading using linear regression.

Thiobarbituric reactive substances (TBARS)

The TBARS assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) which forms an MDA-TBA_2 adduct that absorbs strongly at 532 nm (Spanier and Traylor, 1991). For the TBARS assay, the plasma samples were serially diluted to obtain the optimal dilution at which concentration had the lowest coefficient of variation among measurements on the same sample. Duplicate samples were assayed. The concentrations of TBARS were expressed in terms of malonylaldehyde (MDA) equivalents using a 1/5 dilution.

Mitochondrial genome mutation analysis

Since only the quail and guineafowl had publicly available whole mtGenome sequences, the turkey and budgie sequences developed in this project and described in Chapter 3 and 4 respectively were used in the SNP analysis. The control region or D-loop, and 16S rRNA were targeted for SNP analysis because they were most and least likely to be variable (Parsons et al., 1997; Noda et al., 2001). Pulp was obtained from secondary feathers collected from each bird at the same time of blood collection and used to isolate genomic DNA using a minor modification

of the recommended protocol for the DNeasy Tissue Kit (Qiagen, Valencia, CA, Guan et al., 2007). PCR-based resequencing method (Smith et al., 2001) was used to scan for SNPs in all four avian species at the earliest and latest age group. The mtGenome sequences of budgie, guineafowl, quail and turkey (accession number EF450826, NC_006382, NC_003408, and EF153719, respectively) were used to design primers using the computer program Primer 3 (Rozen and Skaletsky, 1997). Because of a duplication of the control region in the budgie mtGenome, only Control Region 2 was scanned for SNPs. The FailSafe^{TM} PCR PreMixes (Epicentre, Madison, WI) were used to optimize the reaction conditions at primer annealing temperature of 56^{o}C. Primers used in the PCR were shown in **Table 5.2**. Following the optimization, PCR was carried out, the amplicons purified and sequenced as previously described (Lin et at., 2006). The sequences were analyzed for SNPs using Phred, Phrap and Consed according to Gordon et al. (1998).

Statistical analysis

The data were analyzed using the General Linear Model (GLM) procedure (SAS Institute, Cary, NC, 2002). In the initial analysis, the two-way ANOVA option of SAS was used to evaluate age-sex, species-sex and species-age differences in PUA, GSH and TBARS. The data are expressed as means $\pm$ standard errors. Additional regression analysis was carried out after ln-transformation of the oxidative stress measurements. Comparisons were considered significant at $P < 0.05$.

5.4 Results and Discussion

The use of blood to determine the levels of the biomarkers allowed assessment of oxidative stress at different ages. The average PUA levels with standard error are presented in **Figures 5.2-5.4**. The effect of species was significant (P<0.05), with quail had the lowest level of PUA, while guineafowl and budgie had the highest. There were significant (P<0.05) sex effects on PUA in turkey and budgie, with the male turkey and female budgie had higher levels of PUA, but the effect of the interaction between sex and species was not significant (**Figures 5.2**). Age also affected PUA significantly (P<0.05). The PUA level decreased with age as it was significantly higher (P<0.05) at 10 and 30 wks-of-age than at the other two ages. However, there was no significant effect of the interaction between sex and age (**Figure 5.3**). The effect of the interaction between species and age was significant (P<0.05) as well. For example, as shown in **Figure 5.4**, quail had the lowest PUA value in all four age groups, and guineafowl had the highest, except at 30 wks-of-age the PUA level of budgie was the highest.

The GSH levels are presented in **Figures 5.5-5.7**. Similar as PUA, species had significant effect on the level of GSH. As shown in **Figure 5.5**, guineafowl had significantly (P<0.05) higher levels of GSH, but lower in budgie. There was no significant effect of sex or the interaction between sex and species on GSH levels in all the birds (**Figure 5.5**). However, age affected the level of GSH significantly (P<0.05) as GSH was lowest at 55 wks-of-age, but higher at 30 and 80 wks-of-age (**Figure 5.6**). The effect of the interaction between species and age was also significant (P<0.05) on the level of GSH. For instance, at 10 wks-of-age, guineafowl had a significantly higher level of GSH than quail and turkey. At 30 wks-of-age, the GSH concentration in the budgie was lower than in the guineafowl, quail, and turkey. Similarly, the

GSH concentrations in quail and budgie were lower than in the guineafowl and turkey at 55 wks-of-age (**Figure 5.7**).

The levels of lipid peroxidation shown by the TBARS measurement are presented in **Figures 5.8-5.10**. As expected, species affected TBARS significantly (P<0.05). In quail, in contrast to PUA and GSH levels, TBARS were significantly higher (P<0.05) than other birds. There were no significant effects of the interaction between sex and species, but in turkey, female birds had significant (P<0.05) higher level of TBARS than in males (**Figure 5.8**). Age also had significant effect (P<0.05), and the level of TBARS was highest at 55 wks-of-age and lowest at 30 wks-of-age (**Figure 5.9**). The effect of the interaction between species and age on the level of TBARS was significant (P<0.05). At 10 wks-of-age, there was a negative correlation between the level of TBARS and longevity with quail having the highest level of TBARS and turkey having the lowest. At 30 wks-of-age, the concentration of TBARS in quail was significantly lower than in the other birds, but highest at 55 wks-of age. At 80 wks-of-age, differences among guineafowl, quail, and turkey in the level of TBARS were not significant (**Figure 5.10**).

The effect of body weight on oxidative stress is presented in **Table 5.3**. Strong and significant (P<0.05) negative correlations were observed between body weight and GSH in guineafowl, while positive correlation were found in male quail for GSH, and in male guineafowl for PUA.

A total of 30 SNPs were detected in budgie, guineafowl and turkey, but none in quails (**Table 5.4**). These SNPs were the only ones found in all ages evaluated in the four species. In the turkey, a total of three SNPs, two in the 16S rRNA at nucleotide positions 1,623 (A-C) and 1,951 (A-T), and at position 15,953 (C-T) in the D-loop were detected as previously described in

Chapter 3. Two SNPs were detected in the 16S rRNA of the budgie, a C-T transition at nucleotide positions 2,218 and a deletion at 1,822 according to the budgie mtGenome (GenBank accession number: EF450826). In Control Region 2, 20 SNPs, all of them were C-T or A-G transitions, were detected as previously described in Chapter 4. A total of five haplotypes were derived from these SNPs identified in that region with haplotypes 1, 2, 3 had much higher distribution frequencies (**Table 4.4**). Five SNPs were detected in the guineafowl that included three in the D-loop and two in 16S rRNA. The SNPs formed three haplotypes, one of which, GFHAP2 was the most frequent in the guineafowls analyzed here (**Table 5.5**).

As expected, the biomarkers used to assess oxidative stress were not consistent across ages and species. This inconsistency was the primary rational for our use of multiple biomarkers as predictors of oxidative stress. This is consensus with literature reports that a single biomarker may not be a reliable indicator of oxidative stress in vertebrates. For example, a similar inconsistency was reported by Erden-Inal et al. (2001) when they compared enzymatic antioxidant activities and lipid peroxidation in human at different ages. Catalase, glutathione peroxidase, and TBARS increased at older ages, but superoxide dismutase decreased. In a subsequent study, Erden-Inal et al. (2002) found that total GSH, including reduced and oxidized, had a significantly negative correlation with aging, but the oxidized GSH had a positive correlation. In this study, the inconsistency could be due to the effects of several factors. Both ANOVA and regression analysis showed that species, sex, age and body weight significantly affected oxidative stress. Among these factors, strain, age, and the interaction between strain and age appeared to have a higher effect on differences in oxidative stress.

Though several SNPs were detected in three of the species evaluated, variation did not increase with age, which makes the debate about the association of mtDNA mutations with

lifespan of animals still ambiguous. Supporting one side, several studies have showed that tissue-specific mutations accumulate as a function of age (Khrapko et al., 1997; Michikawa et al., 1999). On the other hand, a recent study by Vermulst et al. (2007) reported that mtDNA mutations appeared to be independent of lifespan of mice. They found the *in vivo* frequency of single-base pair mutations in the mtGenome in mice to be more than ten times lower than previously reported.

There are four explanations for the absence of SNPs associated with age in this dissertation study. First, we only scanned two regions of the mtGenome, D-loop and 16S rRNA. More SNPs may be found in other regions, especially the tRNA regions, which have been reported to have the most SNPs (Kujoth et al., 2007). Second, till now, all the data collected were about or less than one and half years of age. As the lifespan of the birds studied here are at least 3-4 years, the birds now were still considered to be young or just at middle age, especially for birds can live more than 10 years. As there are thousands of mtGenome in one cell, some mutations may only occur in a slight percentage of mtGenomes, or may even have not arise (Wallace, 1992). So at this stage, the mtDNA mutation has not increased to the level we can detect. Similarly for the oxidative stress level, it may not show us a clear trend with aging process as they were still young. In future study, as the birds continue growing elder, there might be a better hint of the relationship among oxidative stress, mtDNA mutation, aging, and longevity. Third, the mtDNA was isolated from feather plup, which may not be the tissue having most mutations such as skeletal muscle, neuron and liver. For example, Liu et al., (1998) reported different accumulation of mtDNA deletion with age in different human tissues. In that study, the abundance of deletion at position 4977 of human mtGenome was substantially higher in skeletal muscle than in heart and kidney. The last explanation is that, it might be the case that

mtGenome integrity is not associated with age and oxidative stress as other study reported (Vermulst et al. 2007).

In summary, the level of oxidative stress as well as mtDNA variations of birds with diverse longevities was assessed at different ages. Though the indices of oxidative stress we used here showed an inconsistent trend, this was the new report of the basal oxidative status of these avian species which was indicated by GSH, PUA and TBARS. These basal data may be useful resources for future studies involving nutritional regulation or mutants. There were a total of thirty SNPs detected in the four species evaluated, which did not increase with age. The SNPs found here could, however, be useful for the assignment of function to mtGenome, especially the role of mitochondrial genes in variation of economically important phenotypes in birds.

Table 5.1. Animals used in current study.

Species	Month of hatch	Total number (F/M)	Average longevity (year)
Japanese quail	6/2005	40 (20/20)	3-4
Guineafowl	7/2005	18(9/9)	7-12
Turkey	6/2005	28 (11/17)	12.5
Budgie	2/2006[a]	21(10/11)	17.8

[a] Month obtained from the pet store: the age of the budgies were estimated at about 3-6 month at that time.

Table 5.2. PCR primers and expected size (bp) of amplicons used in the D-loop and 16S rRNA SNP analysis in four avian species.

Primer ID[a]	Primers	Length (bp)
TDLF	5'-CCAAGGATTACGGCTTGAAA-3'	1477bp
TDLR	5'-TTAAGCTATGGGGGCTGTTG-3'	
QDLF	5'-AGATCACCAACCCCTGTCTG-3'	1099bp
QDLR	5'-ACCCTGTGGTCTTGCTATGG-3'	
GDLF	5'-ATACCTCCACCCCATTCCTC-3'	894bp
GDLR	5'-TGTTGTGGTAGTGGGGTTTCT-3'	
BDL2F[b]	5'-AAAGCCACCCCATTAAAATC-3'	1993bp
BDL2R[b]	5'-AAGGGTGTGTGGGTGTTTTC-3'	
T16SF	5'-ACAACCAAGCAAAGCGAACT-3'	1111bp
T16SR	5'-ATGGGCTCTTGGAGGAGATT-3'	
Q16SF	5'-CCAAGCAAAGCGGACTAAAG-3'	948bp
Q16SR	5'-ACCGAAAAATGTCGACCAAG-3'	
G16SF	5'-GTGAAAAGCCTACCGAGCTG-3'	994bp
G16SR	5'-ATGGGCTCTTGGAGGAGATT-3'	
B16SF	5'-CCTAAAGCAGCCACCCATAA-3'	916bp
B16SR	5'-TAGAAACCGACCTGGATTGC-3'	

[a] T, Q, G, and B indicated primer sequence from turkey, quail, guineafowl and budgie, respectively, and DL and 16S represent primers for the D-loop and 16S rRNA respectively, of the mitochondrial genome.

[b] Primers from the budgie Control Region 2 of the mitochondrial genome.

Table 5.3. Regression coefficient, coefficient of determination (R^2), and *P* value of the correlation between ln-transformed measurements of indicators of oxidative stress and body weight in birds.

Birds	Biomarkers[a]	Female			Male		
		Regression coefficient[c]	R^2	*P* value	Regression coefficient	R^2	*P* value
Budgie	PUA	NS[b]			NS		
	GSH	NS			NS		
	TBARS	NS			NS		
Guineafowl	PUA	NS			0.51451	0.4789	0.0010
	GSH	-0.31161	0.4061	< 0.001	-0.30444	0.4146	0.0029
	TBARS	NS			NS		
Quail	PUA	NS			NS		
	GSH	NS			19.55181	0.1533	0.0294
	TBARS	NS			NS		
Turkey	PUA	NS			NS		
	GSH	NS			NS		
	TBARS	NS			NS		

[a] Oxidative stress indicators include plasma uric acid (PUA), glutathione (GSH), and thiobarbituric reactive substances (TBARS).

[b] The regression coefficient was not significant ($P>0.05$).

[c] Detailed data were available in Appendices.

Table 5.4. The total number of SNPs observed in four avian species in D-loop and 16S rRNA of the mitochondrial genome.

Species	D-loop	16S rRNA
Budgie	20[a]	2
Guineafowl	3	2
Quail	0	0
Turkey	1	2

[a] For budgie, only Control Region 2 was screened for SNPs.

Table 5.5. Guineafowl control region and 16S rRNA haplotypes and diversity.

ID	Haplotype[a]	Frequency[b]
GFHAP1	-G-T-T-C-A-	0.333
GFHAP2	-A-C-C-T-A-	0.583
GFHAP3	-A-C-C-T-G-	0.083

[a] The SNP position (according to GenBank accession number: NC_006382) were listed correspond to each site as follows: 259, 270, 300, 2760, and 2976, respectively.

[b] Haplotype frequency was determined in a total of 12 guineafowls.

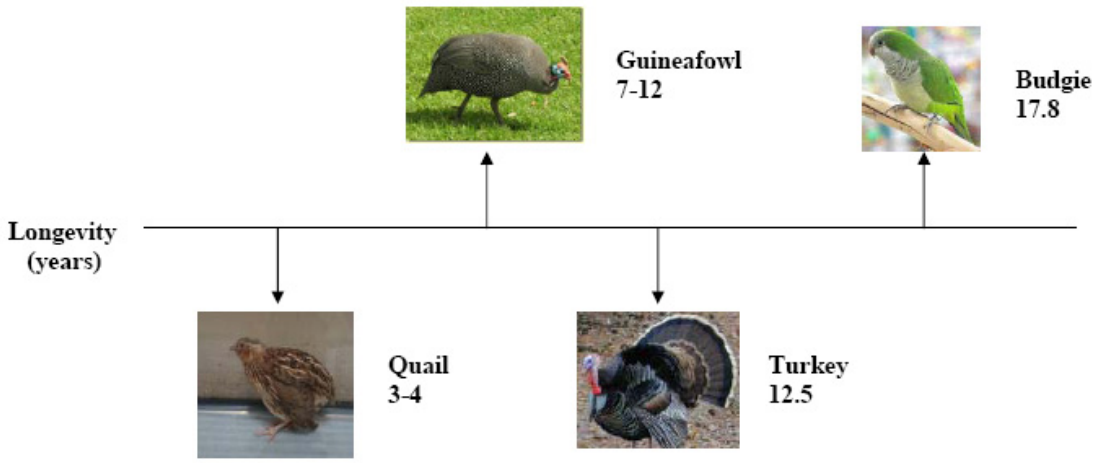


Figure 5.1. A sketch of the longevity in years (not drawn to scale) of different avian species. The number by each species represents the maximum reported life span. For the quail, the numbers represent the range of average lifespan, while for guineafowl, 7 years represent longevity in the wild, and 12 years in captivity.

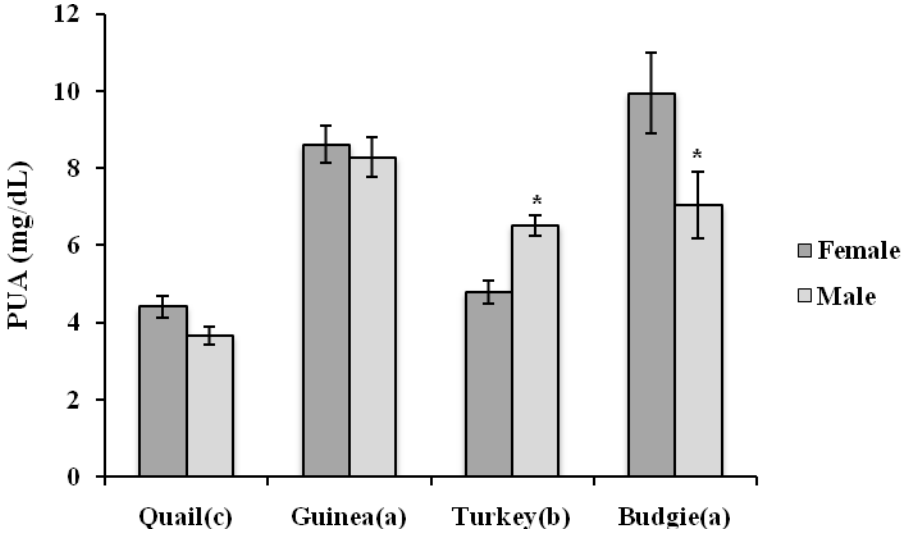


Figure 5.2. Average plasma uric acid (PUA) levels with standard error in quail, guineafowl, turkey, and budgie, respectively.

abc Differences were significant among species ($P < 0.05$).

***** There was significant difference of the PUA level between male and female birds.

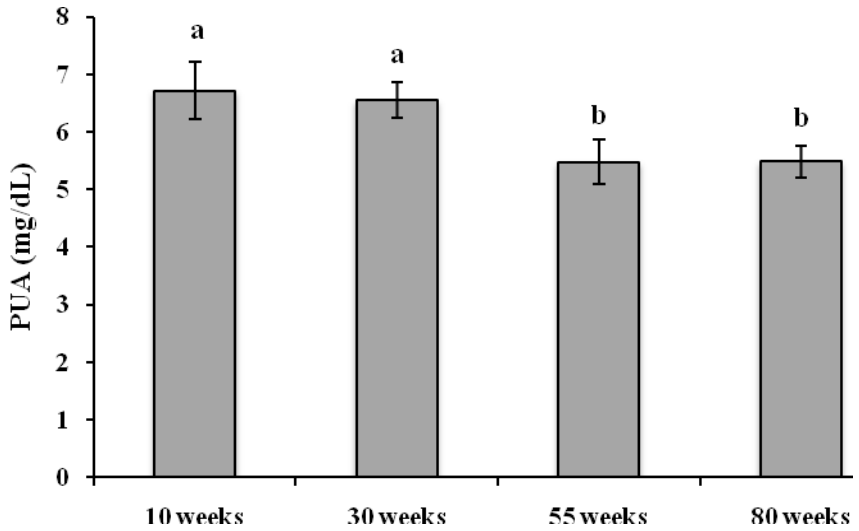


Figure 5.3. Average plasma uric acid (PUA) levels with standard error at 10, 30, 55 and 80 wks-of-age, respectively.

[ab] Differences were significant at different ages ($P < 0.05$).

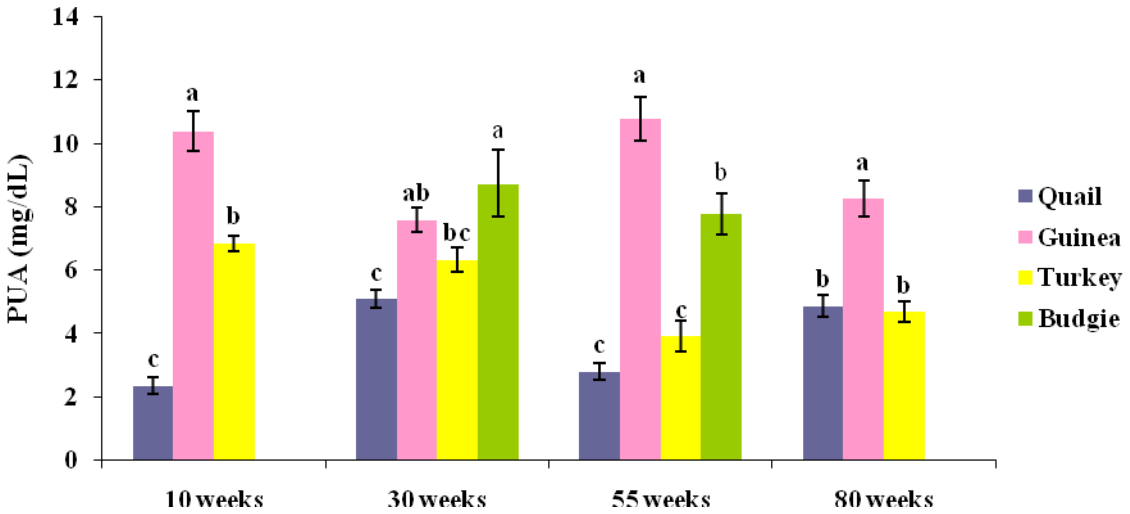


Figure 5.4. Average plasma uric acid (PUA) levels with standard error in different avian species at 10, 30, 55 and 80 wks-of-age, respectively.

[abc] Differences were significant ($P < 0.05$) among different species at one age group.

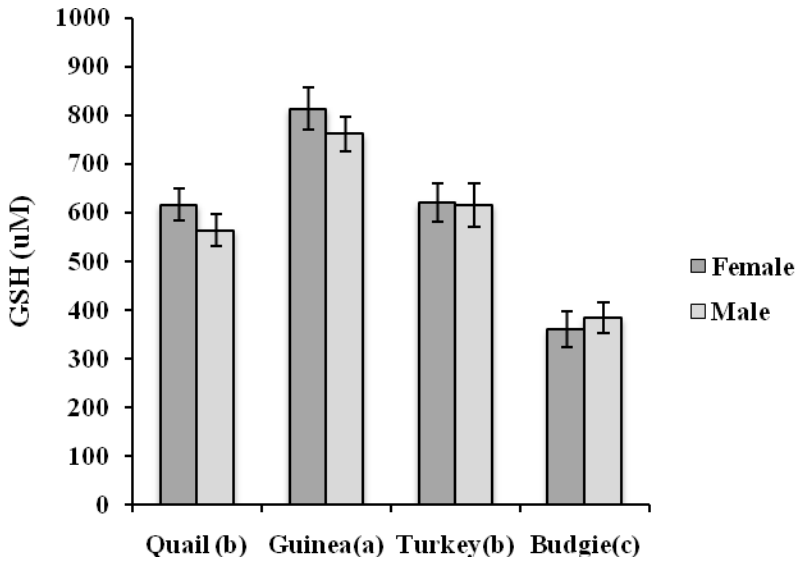


Figure 5.5. Average total glutathione (GSH) levels with standard error in quail, guineafowl, turkey, and budgie, respectively.

[abc] Differences were significant among species ($P < 0.05$).

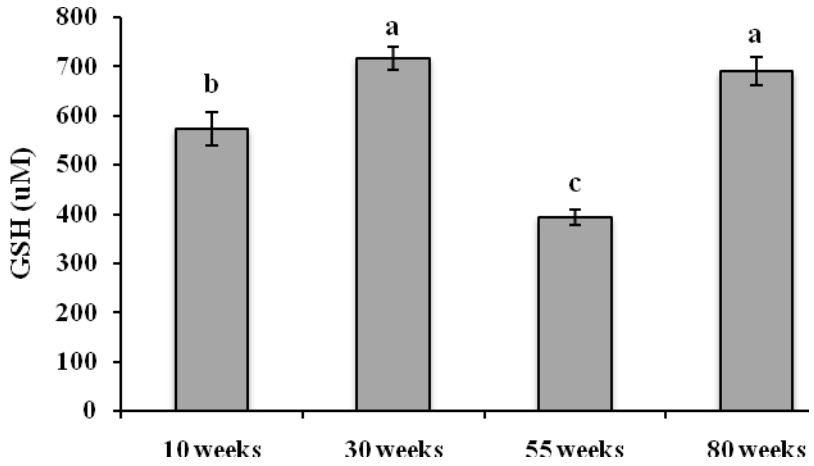


Figure 5.6. Average total glutathione (GSH) levels with standard error at 10, 30, 55 and 80 wks-of-age, respectively.

[abc] Differences were significant at different ages ($P < 0.05$).

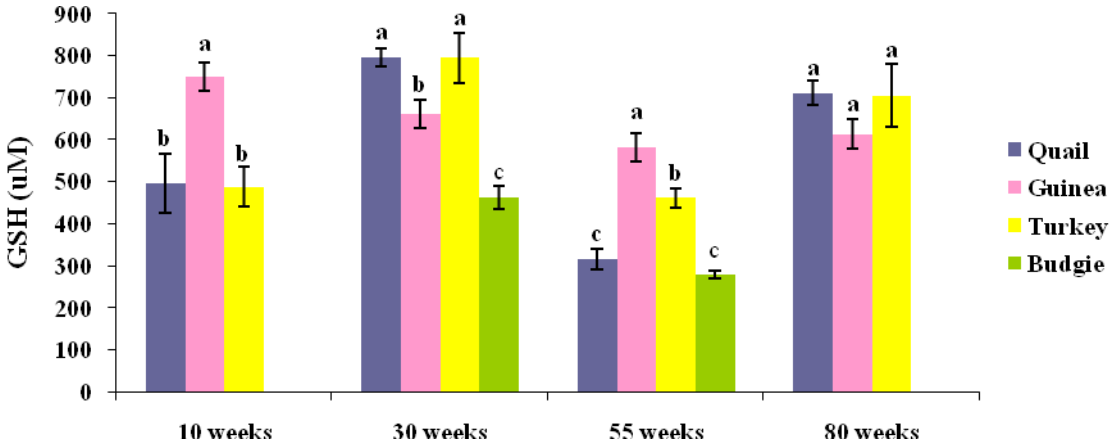


Figure 5.7. Average total glutathione (GSH) levels with standard error in different avian species at 10, 30, 55 and 80 wks-of-age, respectively.

[abc] Differences were significant ($P < 0.05$) among different species at one age group.

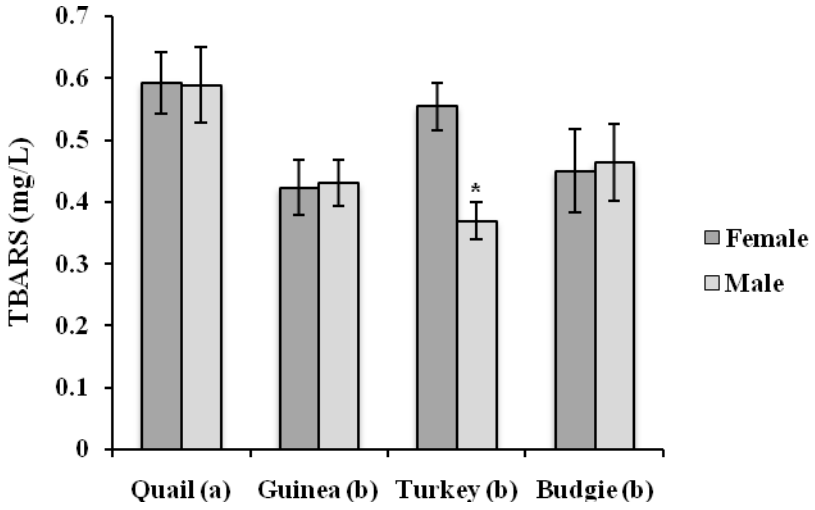


Figure 5.8. Average thiobarbiturate acid reacting substance (TBARS) levels with standard error in quail, guineafowl, turkey, and budgie, respectively.

ab Differences were significant among species ($P < 0.05$).

***** There was significant difference of the PUA level between male and female birds.

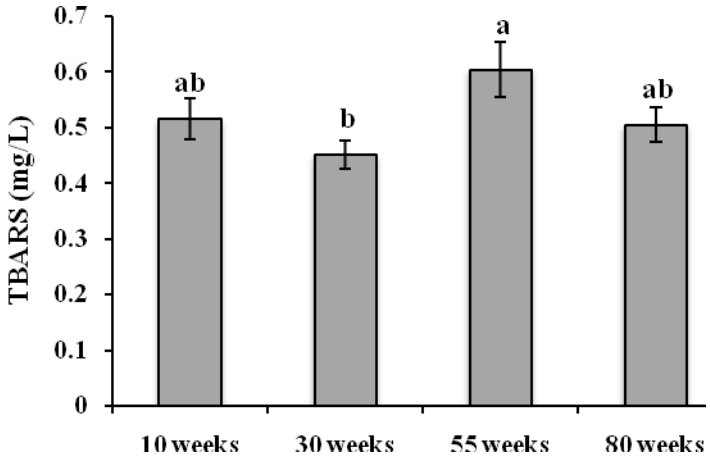


Figure 5.9. Thiobarbiturate acid reacting substance (TBARS) levels with standard error at 10, 30, 55 and 80 wks-of-age, respectively.

[ab] Differences were significant at different ages ($P < 0.05$).

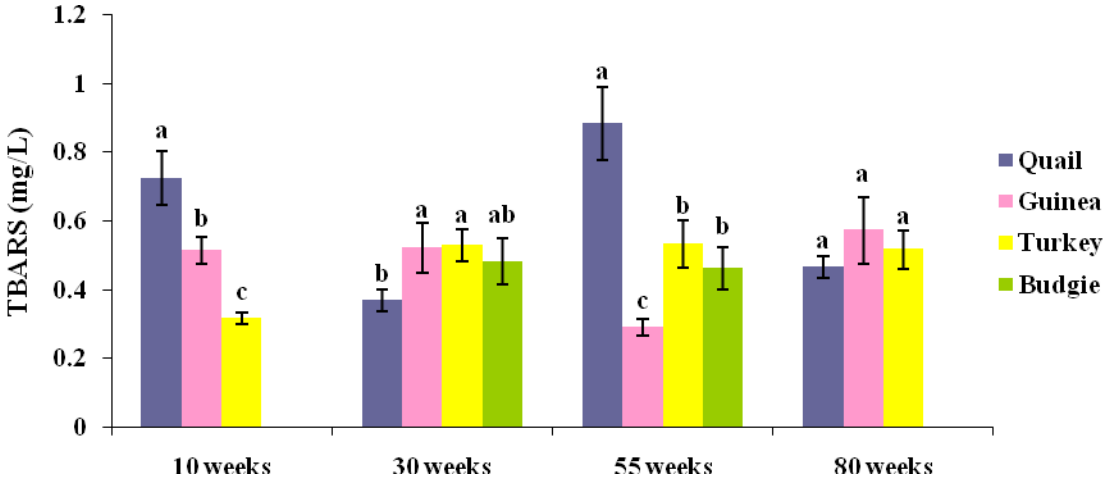


Figure 5.10. Thiobarbiturate acid reacting substance (TBARS) levels with standard error in different avian species at 10, 30, 55 and 80 wks-of-age, respectively.

[abc] Differences were significant ($P < 0.05$) among different species at one age group.

CHAPTER 6

Summary and Conclusion

This dissertation research investigated the hypothesis that oxidative stress is the primary biological mechanism responsible for differences in longevity among birds (budgie, guineafowl, quail, and turkey) and that this difference correlates with mitochondrial genome (mtGenome) integrity both within and among species. The rational for the research is that as the site of a large amount of ROS production, macromolecules in the mitochondria including DNA are believed to be under high levels of oxidative stress. A disproportionate amount of mutations in the mitochondria DNA could signal higher levels of oxidative stress.

Specific findings:

1. In the preliminary investigation to develop a reference using the chicken mtGenome, a total of 113 single nucleotide polymorphisms (SNPs) was identified in the whole mtGenome by both experimental and *in silico* tools. The *in silico* analysis revealed a total of 91 SNPs, while experimental analysis detected 41 SNPs. Nineteen of the 91 SNPs detected in the *in silico* analysis were also observed in the experimental SNP analysis. All the 41 SNPs observed by experimental analysis formed 17 haplotypes.
2. The sequence of the turkey mtGenome, 16,967 bp in length, was obtained. The relatedness among different turkey variaties were evaluated by using one SNP in control region and other two SNPs in 16S rRNA. Mitochondrial genome-based phylogenetics anaylsis showed that turkey was most closely related to chicken and quail.

3. The complete budgie mtGenome, 18,193 bp in length, was sequenced. A special rearrangement, control region duplication, was detected in budgie mtGenome. A total of 22 SNPs were detected, with 2 in 16S rRNA and 20 in one of the control regions. The phylogenetic analysis showed that budgie was closest to kakabo.
4. By using three biomarkers including TBARS, PUA and GSH, it appeared that the change in oxidant and antioxidant status varied with species and was significantly affected by age.
5. The SNPs observed in all four species had no correlation with age.

Future work:

This study used PCR-based methods to identify mutations in mtGenome, developed the complete mtGenome of two avian species, and detected SNPs in different regions of mtGenome in several species. The basal oxidative stress in budgie, guineafowl, quail and turkey was also established. However, the association among oxidative stress, mtGenome integrity at different ages and longevity remains unclear. Specific investigations that could help answer this question include:

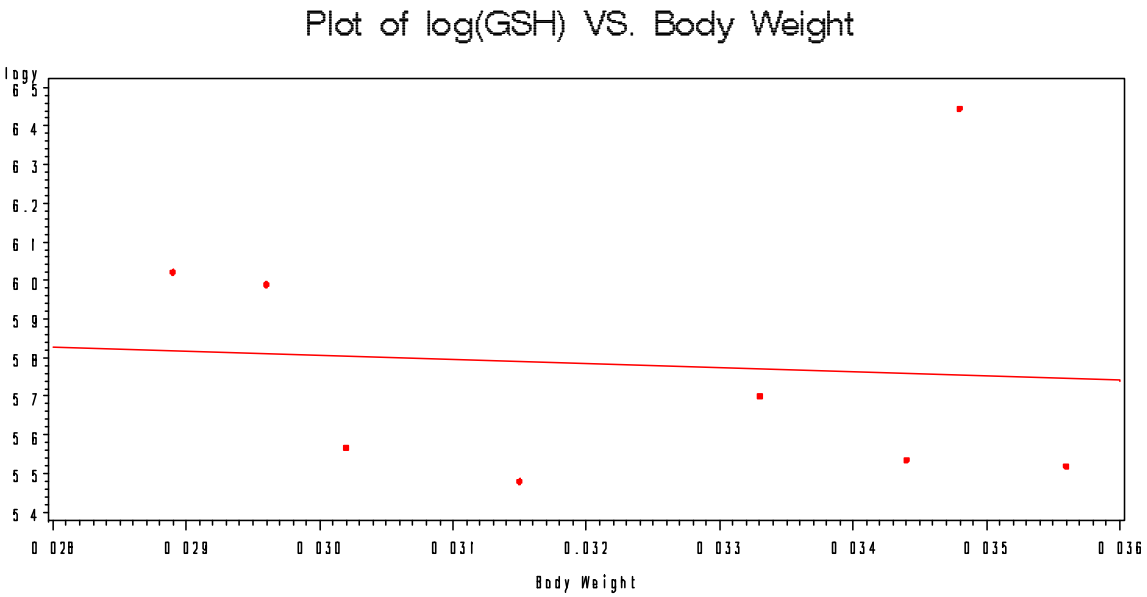
1. Evaluate of oxidative stress by using later measurements in birds after reproductive age.
2. Use tissues from other organs than feather pulp, such as skeletal muscle, to assess the effect of oxidative stress on mitochondrial DNA mutation.
3. Conduct genome-wide scan to screen regions other that the D-loop and 16S rRNA to assess the link between age and mitochondrial DNA integrity.

APPENDIX

Plots of Regression Coeffients of the Relationships between Body weight and Biomarlers of Oxidative stress

LIST of APPENDICES

Appendix 1. Plot of ln(GSH) and body weight in female budgies



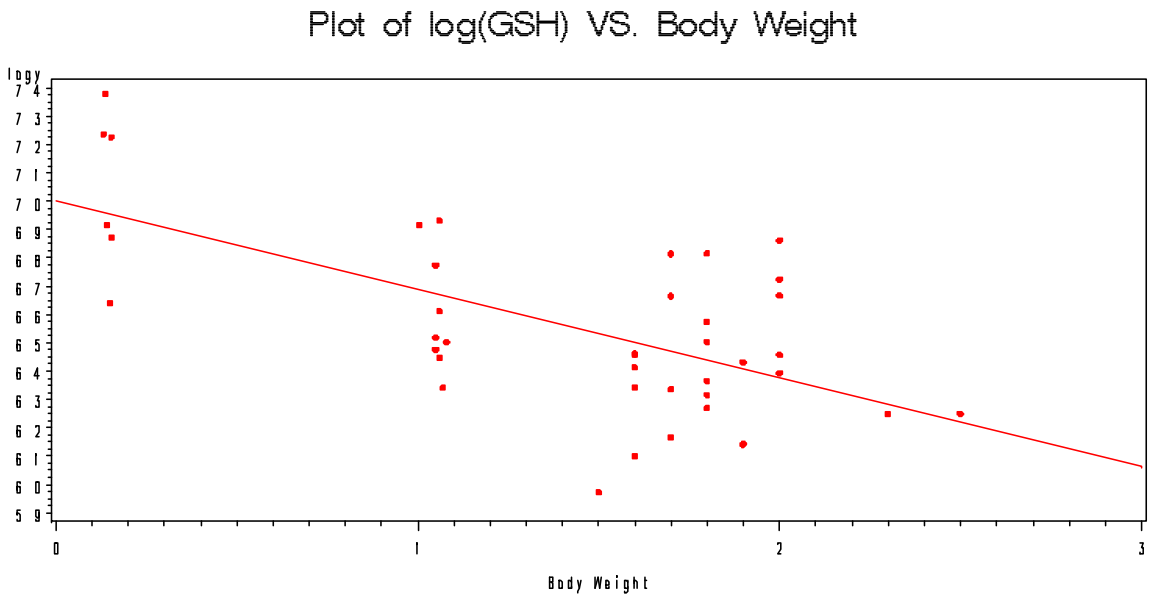
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

$y = -10.50203x + 6.12200$

$R^2 = 0.0063$, $P = 0.8520$.

No significant correlation was found between ln(GSH) and body weight in female budgies.

Appendix 2. Plot of ln(GSH) and body weight in female guineafowls



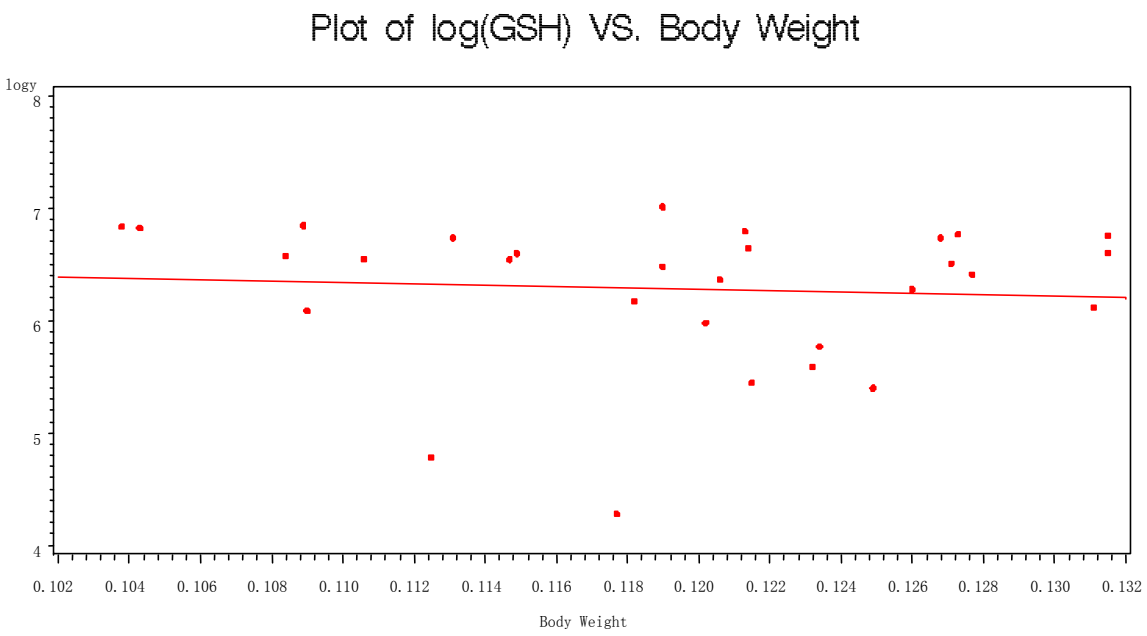
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

$y = -0.31161x + 7.00104$

$R^2 = 0.4061$, $P < 0.001$.

There was a significant negative correlation found between ln(GSH) and body weight in female guineafowls.

Appendix 3. Plot of ln(GSH) and body weight in female quails



Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

y = -5.76392x + 6.97273

R^2 = 0.0051, P = 0.7087.

No significant correlation was found between ln(GSH) and body weight in female quails.

Appendix 4. Plot of ln(GSH) and body weight in female turkeys

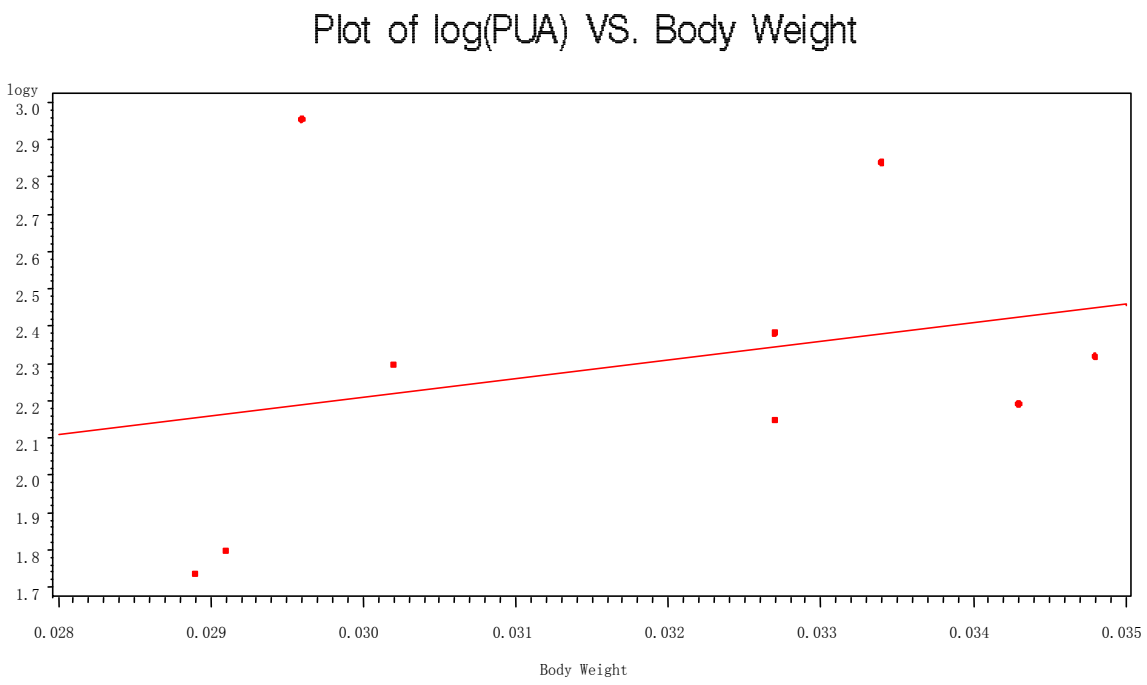
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

$y = 0.05246x + 6.17456$

$R^2 = 0.0196$, $P = 0.4603$.

No significant correlation was found between ln(GSH) and body weight in female turkeys.

Appendix 5. Plot of ln(PUA) and body weight in female budgies



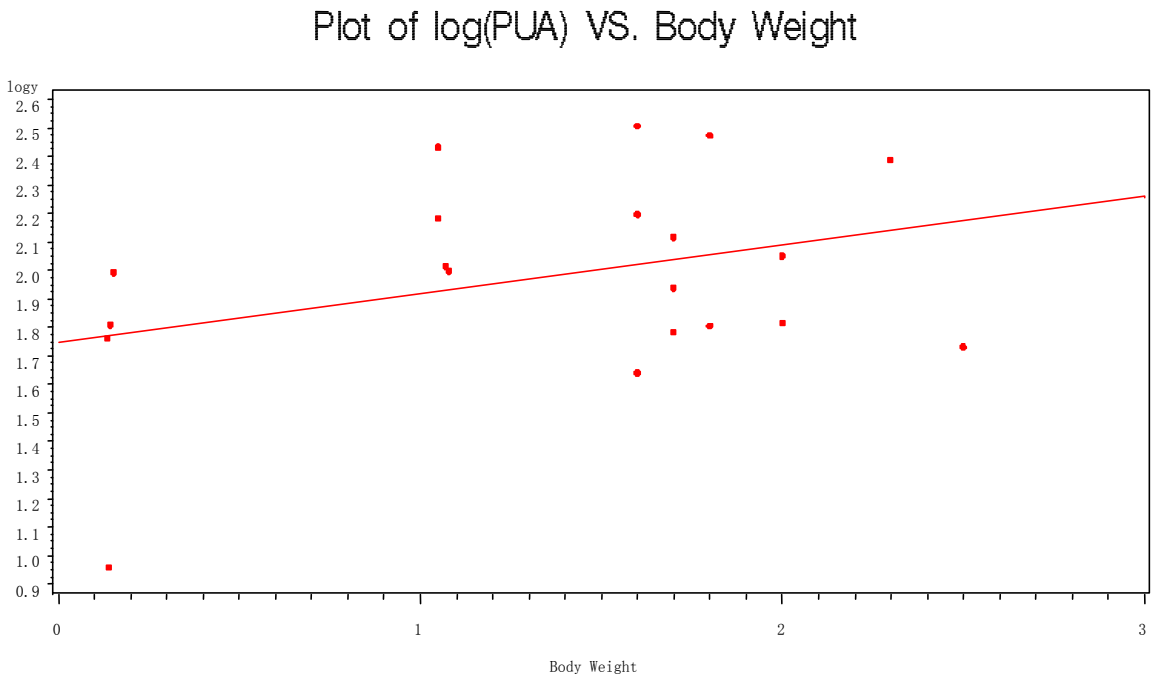
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

y = 50.24886x + 0.70224

R^2 = 0.0806, P = 0.4592.

No significant correlation was found between ln(PUA) and body weight in female budgies.

Appendix 6. Plot of ln(PUA) and body weight in female guineafowls



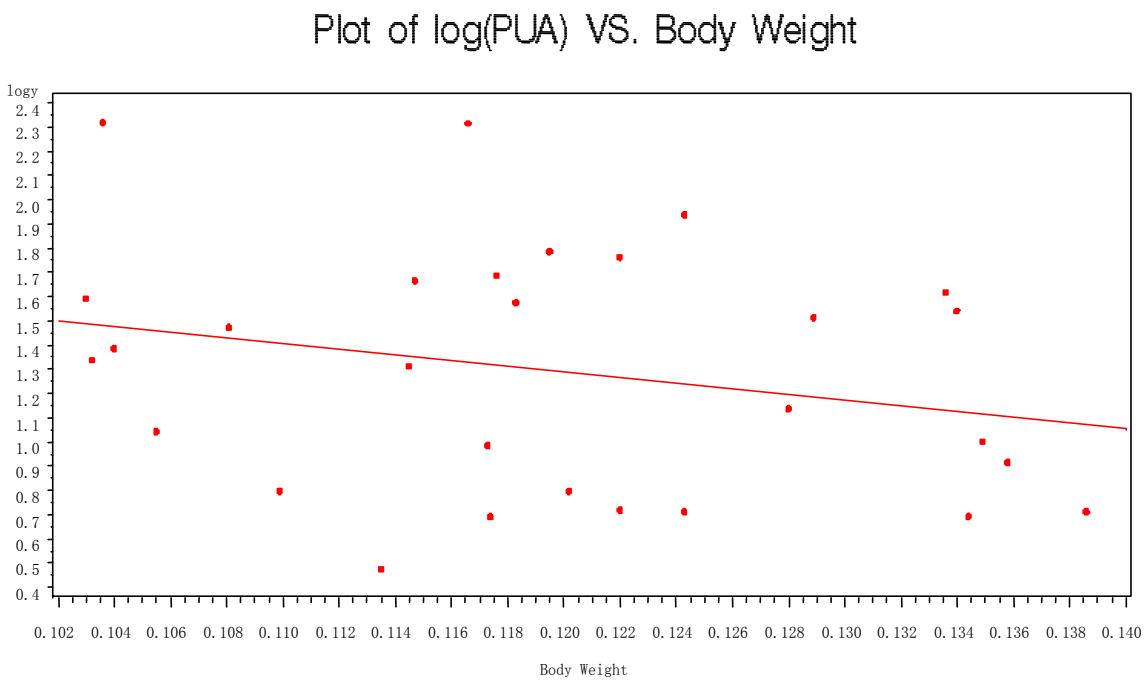
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

$y = 0.17005x + 1.74989$

$R^2 = 0.1225$, $P = 0.1303$.

No significant correlation was found between ln(PUA) and body weight in female guineafowls.

Appendix 7. Plot of ln(PUA) and body weight in female quails



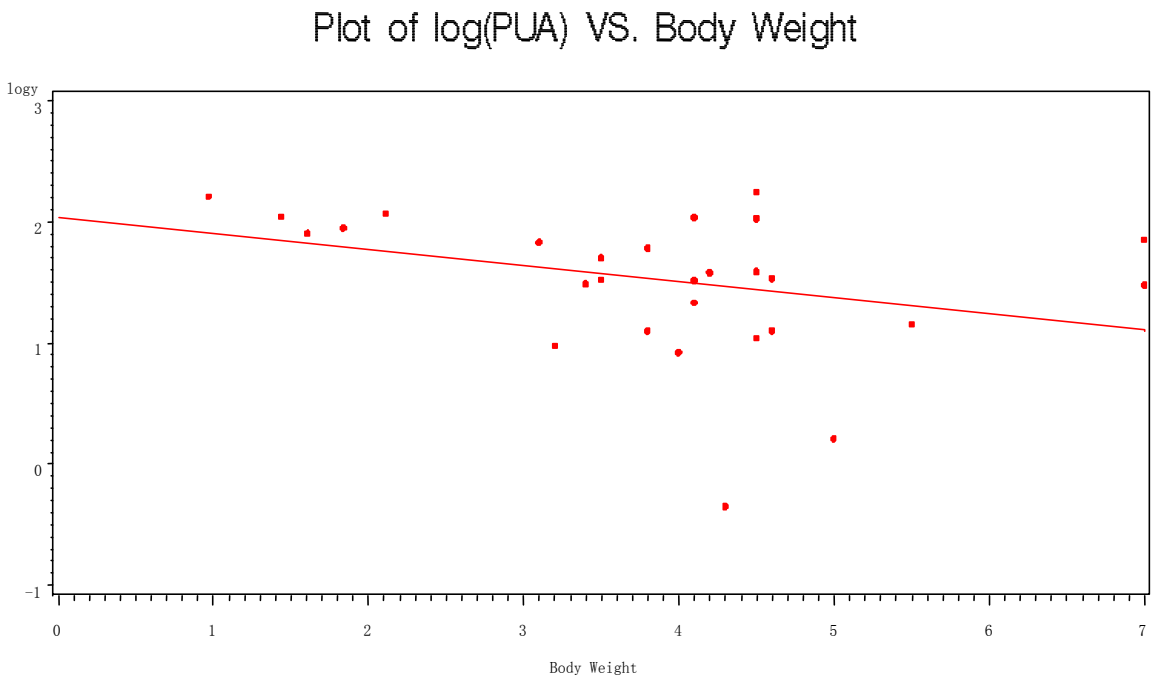
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

y = -11.71789x + 2.69506

R^2 = 0.0643, P = 0.1844.

No significant correlation was found between ln(PUA) and body weight in female quails.

Appendix 8. Plot of ln(PUA) and body weight in female turkeys



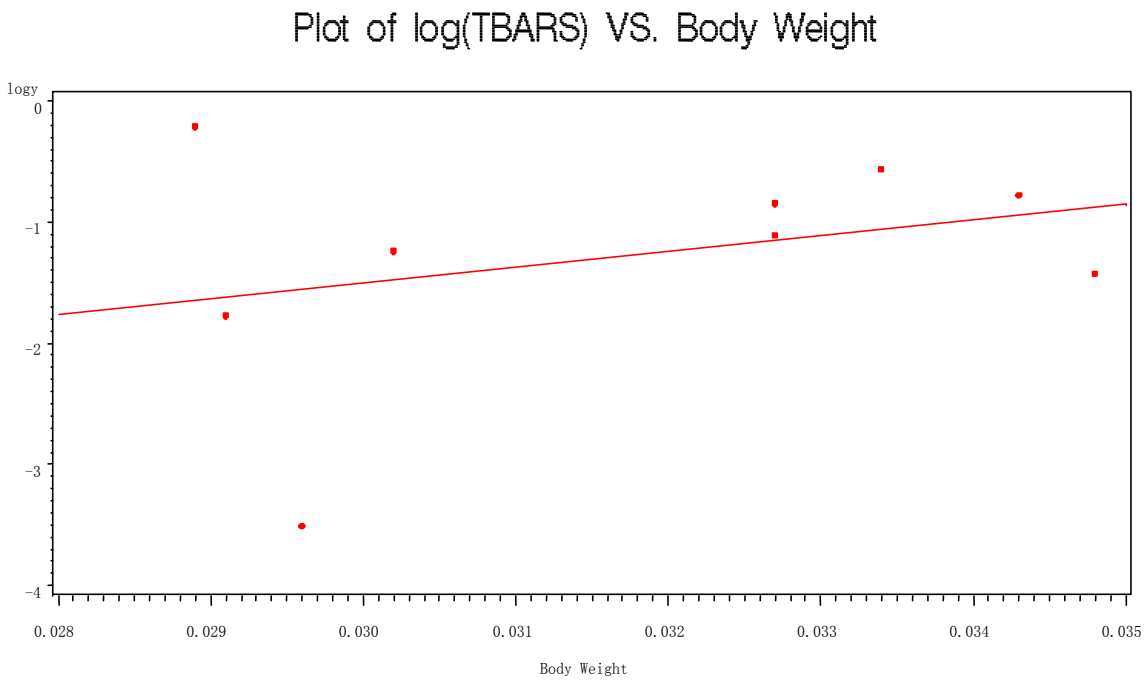
For linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

y = -0.13226x + 2.03160

R^2 = 0.1004, P = 0.0940.

No significant correlation was found between ln(PUA) and body weight in female turkeys.

Appendix 9. Plot of ln(TBARS) and body weight in female budgies



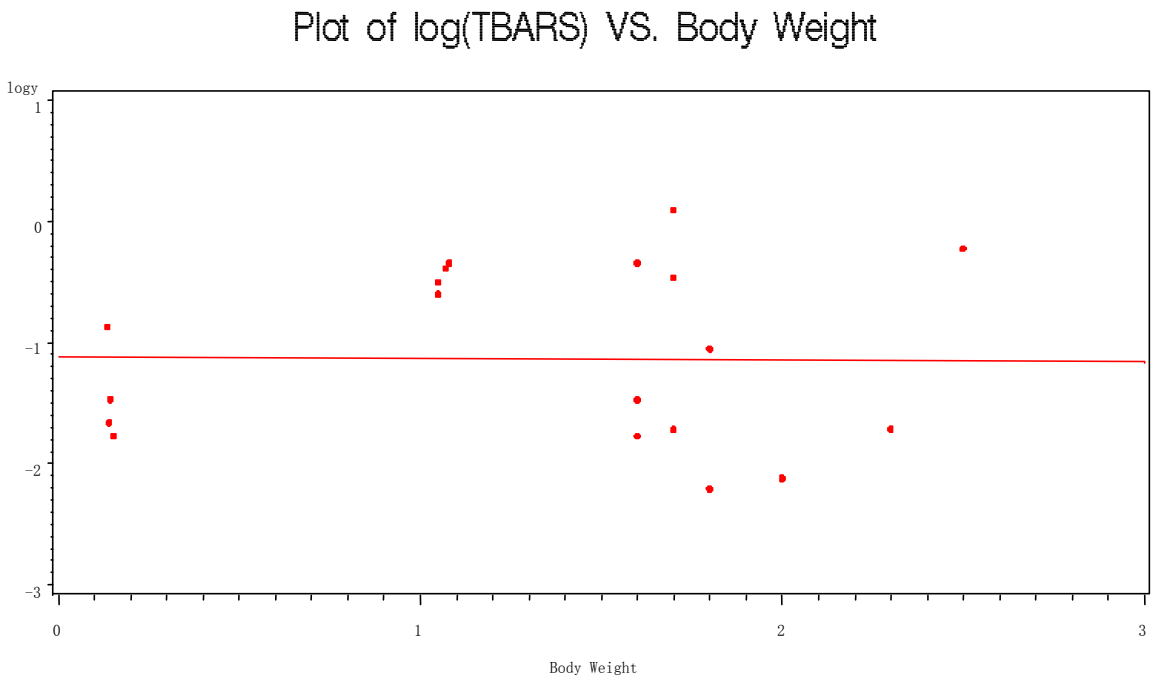
Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = 131.17217x - 5.43571

R^2 = 0.0994, P = 0.4085.

No significant correlation was found between ln(TBARS) and body weight in female budgies.

Appendix 10. Plot of ln(TBARS) and body weight in female guineafowls



Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = -0.01060x - 1.12060

R^2 = 0.0001, P = 0.9651.

No significant correlation was found between ln(TBARS) and body weight in female guineafowls.

Appendix 11. Plot of ln(TBARS) and body weight in female quails

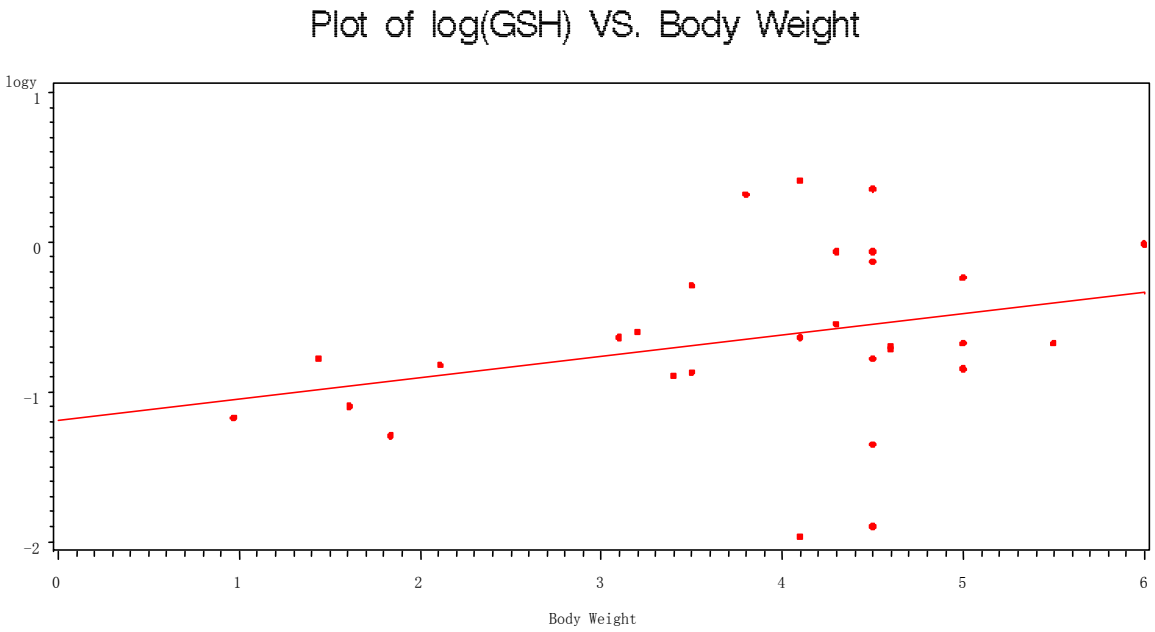
Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = 6.94007x - 1.46045

R^2 = 0.0089, P = 0.3875.

No significant correlation was found between ln(TBARS) and body weight in female quails.

Appendix 12. Plot of ln(TBARS) and body weight in female turkeys



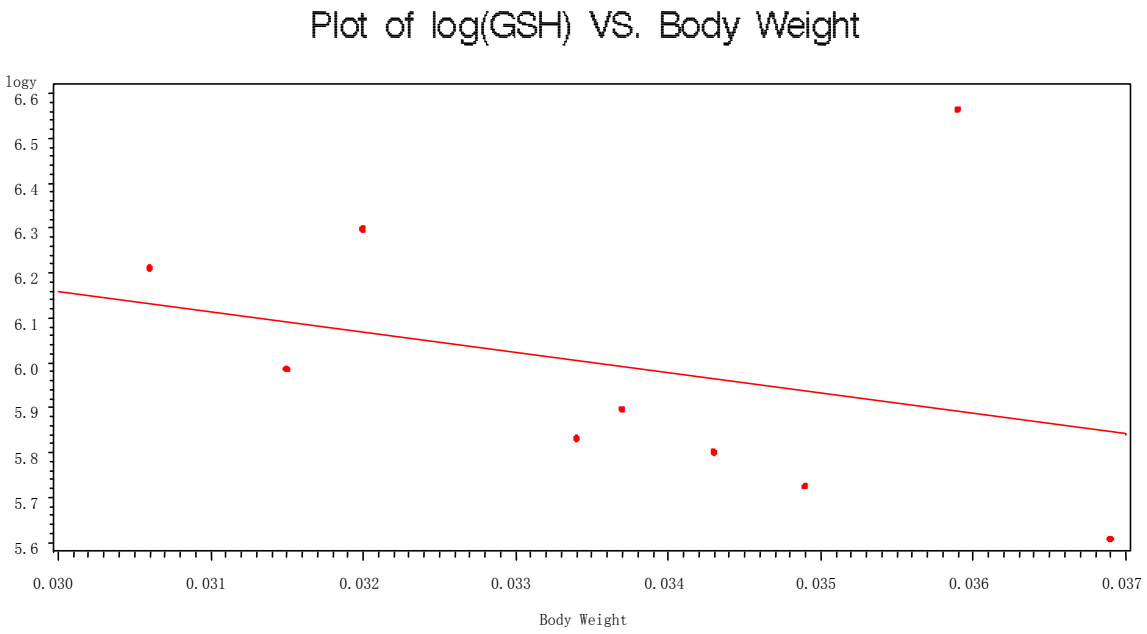
Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = 0.14157x - 1.18853

R^2 = 0.0898, P = 0.1144.

No significant correlation was found between ln(TBARS) and body weight in female turkeys.

Appendix 13. Plot of ln(GSH) and body weight in male budgies



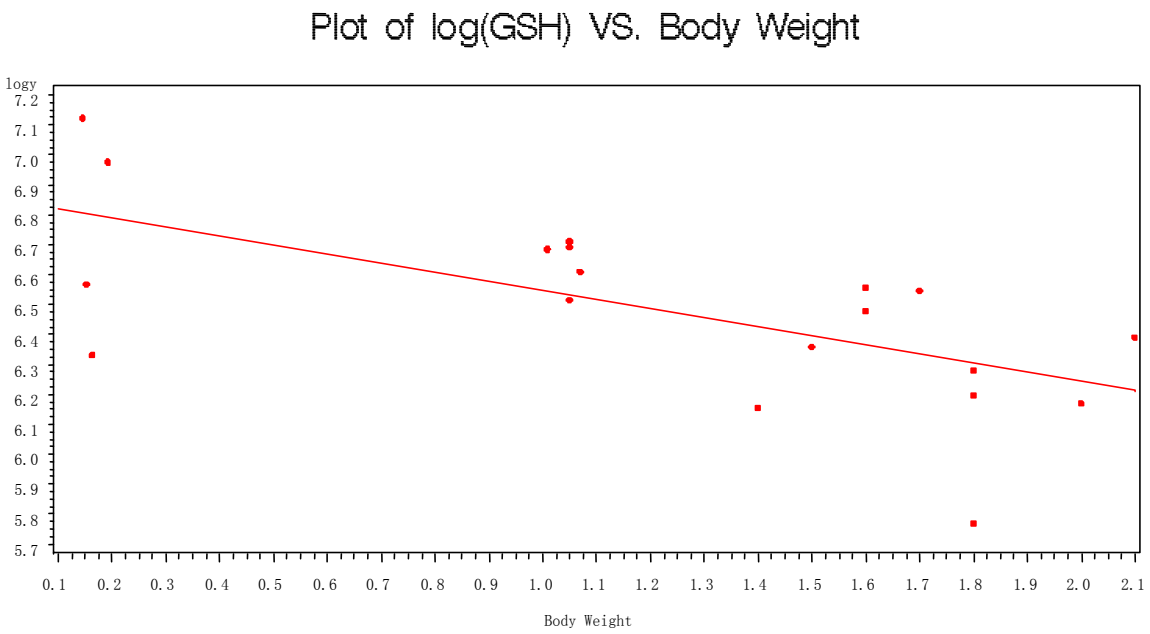
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

y = -44.85598x + 7.50313

R^2 = 0.0909, P = 0.4305.

No significant correlation was found between ln(GSH) and body weight in male budgies.

Appendix 14. Plot of ln(GSH) and body weight in male guineafowls



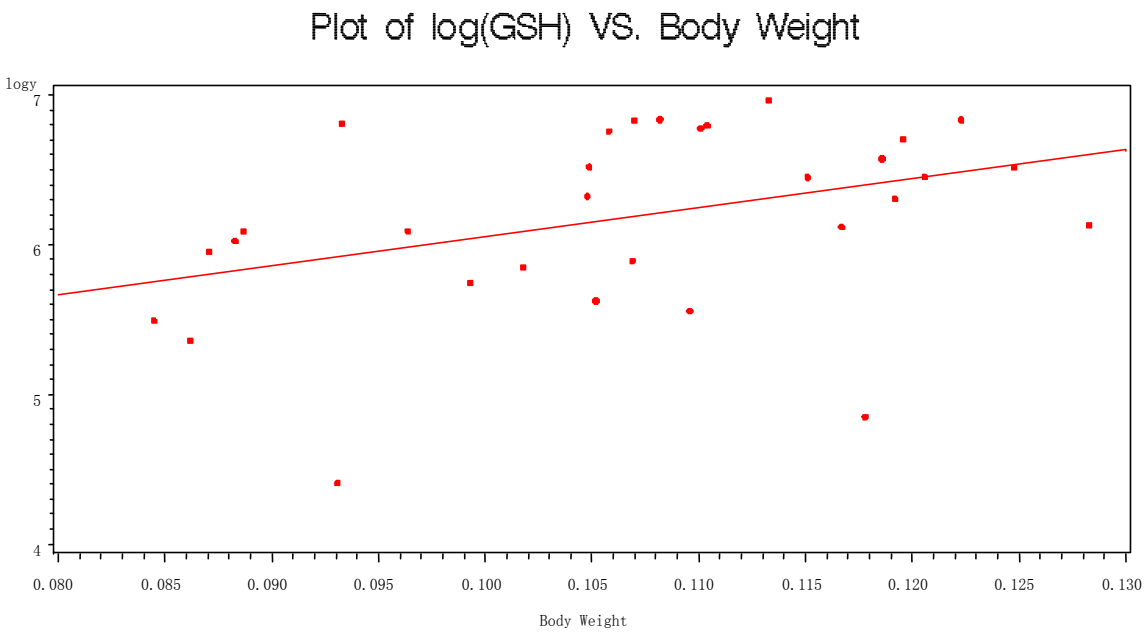
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

y = -0.30444x + 6.85171

R^2 = 0.4146, P = 0.0029.

There was a significant negative correlation found between ln(GSH) and body weight in male guineafowls.

Appendix 15. Plot of ln(GSH) and body weight in male quails



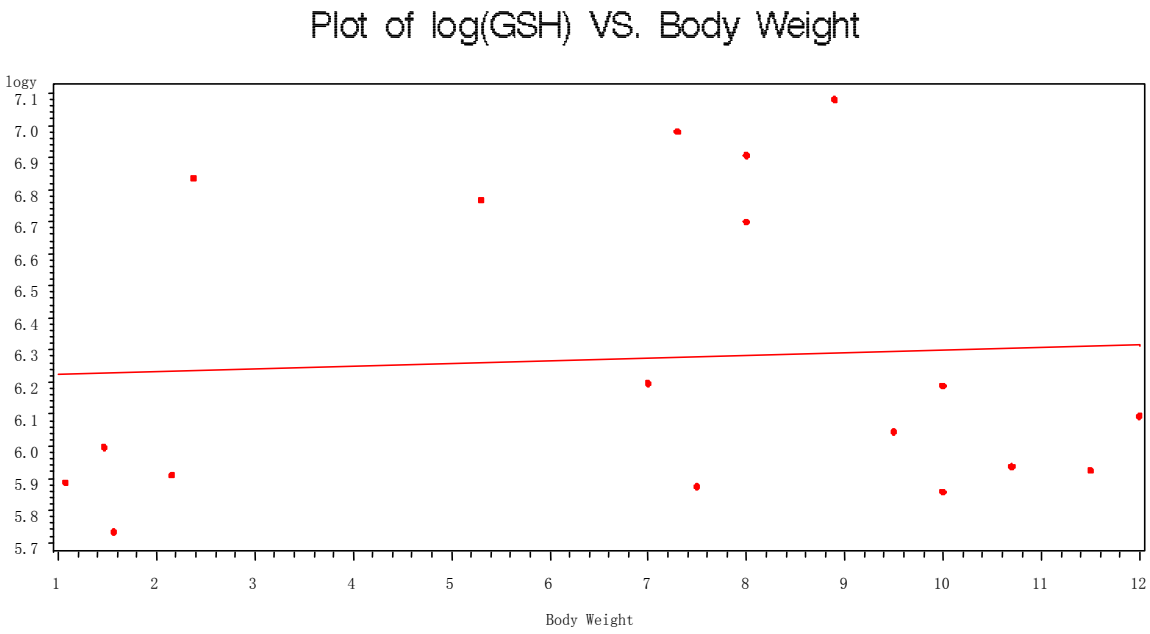
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

$y = 19.55181x + 4.09633$

$R^2 = 0.1533$, $P = 0.0294$.

There was a significant positive correlation found between ln(GSH) and body weight in male quails.

Appendix 16. Plot of ln(GSH) and body weight in male turkeys



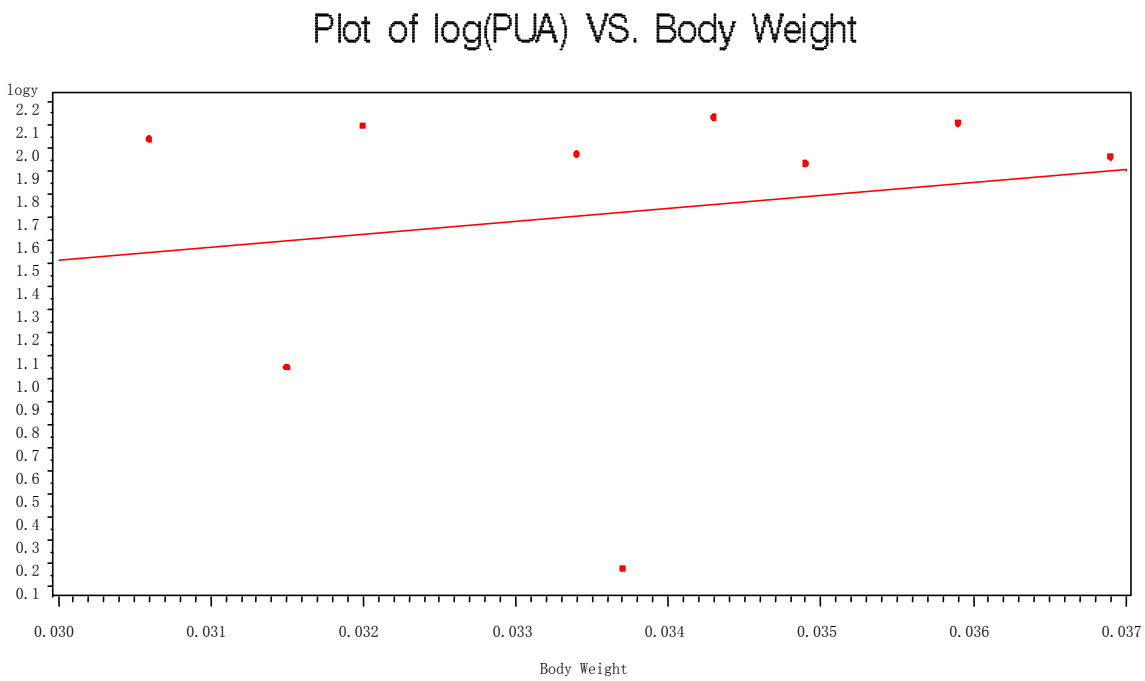
Linear regression model: y is ln-transformed measurement of GSH, and x is body weight:

$y = 0.00814x + 6.21715$

$R^2 = 0.0043$, $P = 0.7970$.

No significant correlation was found between ln(GSH) and body weight in male turkeys.

Appendix 17. Plot of ln(PUA) and body weight in male budgies



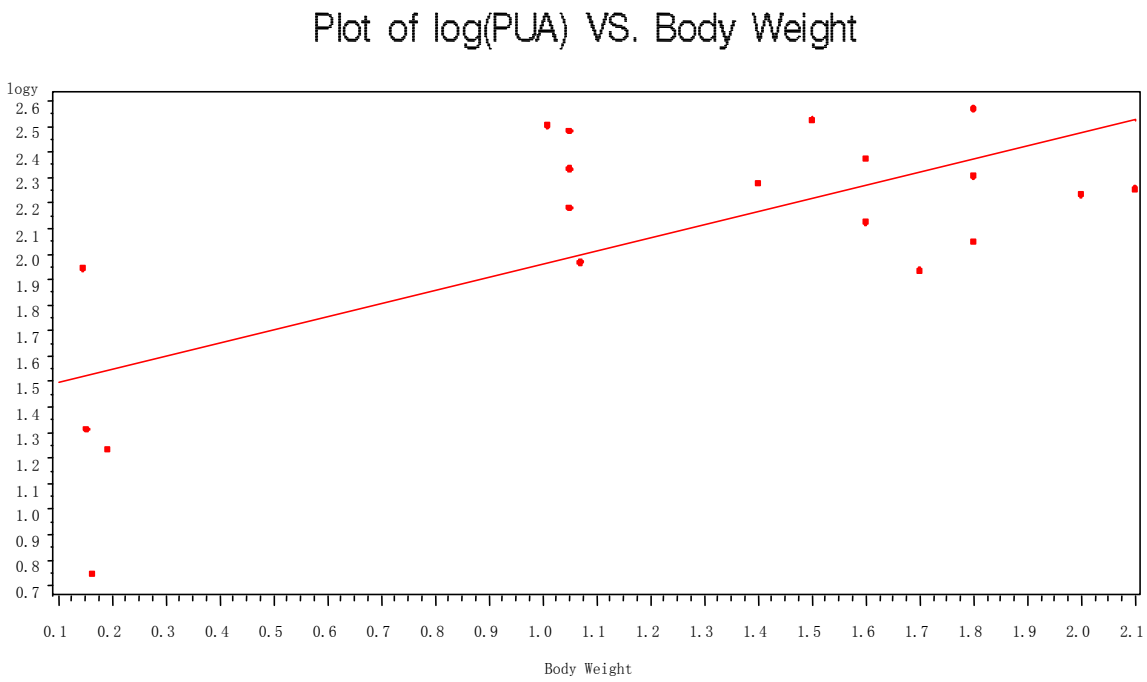
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

y = 55.33811x - 0.14327

R^2 = 0.0295, P = 0.6586.

No significant correlation was found between ln(PUA) and body weight in male budgies.

Appendix 18. Plot of ln(PUA) and body weight in male guineafowls



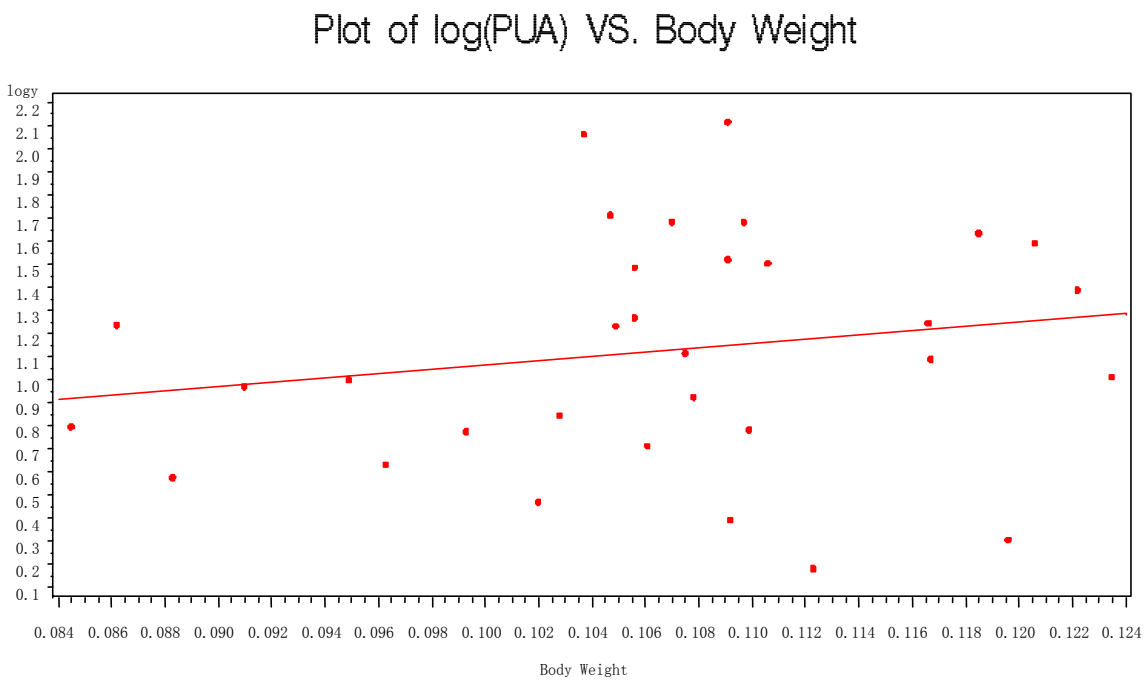
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

$y = 0.51451x + 1.44518$

$R^2 = 0.4789$, $P = 0.0010$.

There was a significant positive correlation found between the ln(PUA) and body weight in male guineafowls.

Appendix 19. Plot of ln(PUA) and body weight in male quails



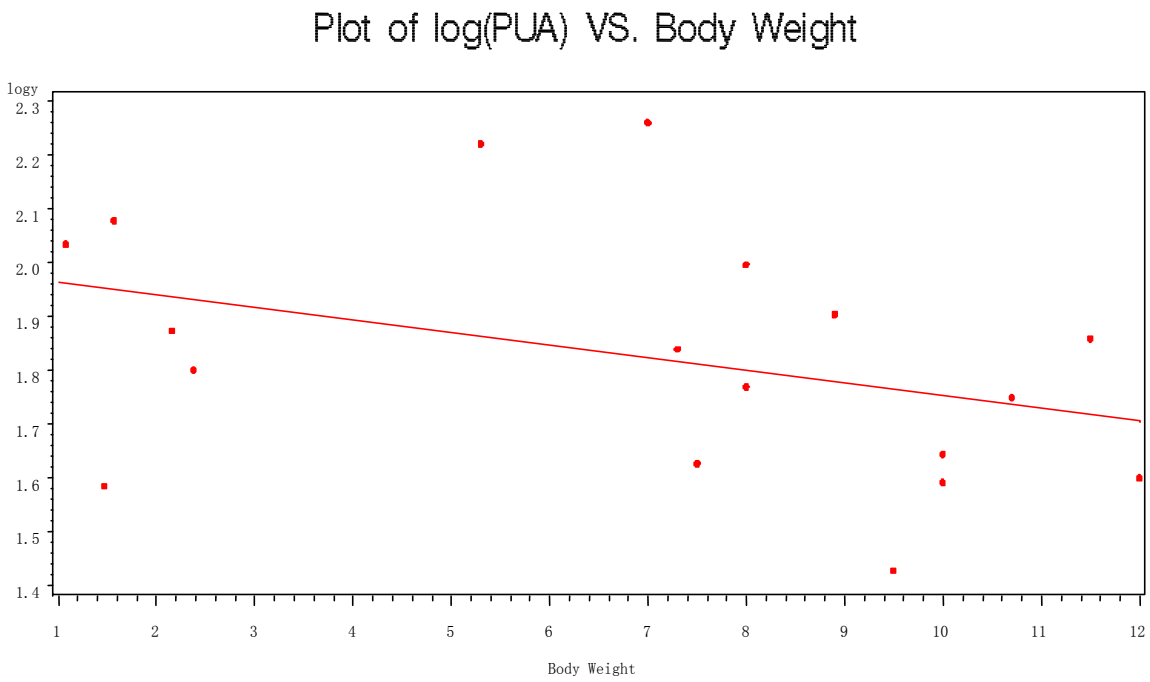
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

$y = 9.36342x + 0.12723$

$R^2 = 0.0363$, $P = 0.2962$.

No significant correlation was found between ln(PUA) and body weight in male quails.

Appendix 20. Plot of ln(PUA) and body weight in male turkeys



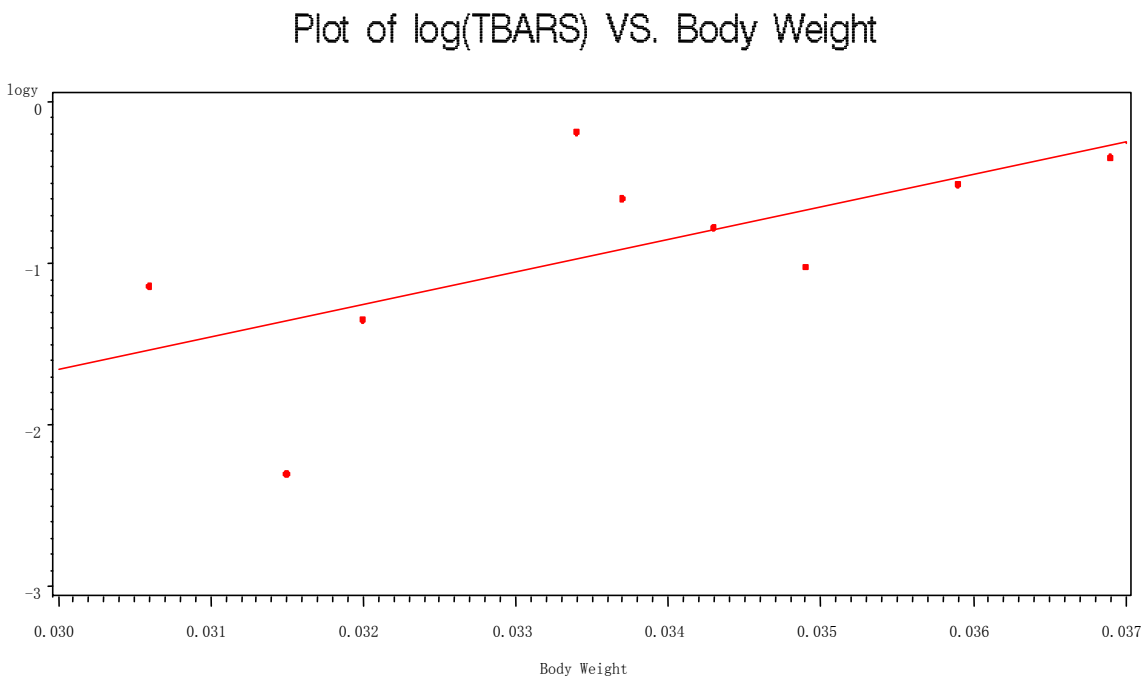
Linear regression model: y is ln-transformed measurement of PUA, and x is body weight:

y = -0.02327x + 1.98654

R^2 = 0.1405, P = 0.1254.

No significant correlation was found between ln(PUA) and body weight in male turkeys.

Appendix 21. Plot of ln(TBARS) and body weight in male budgies



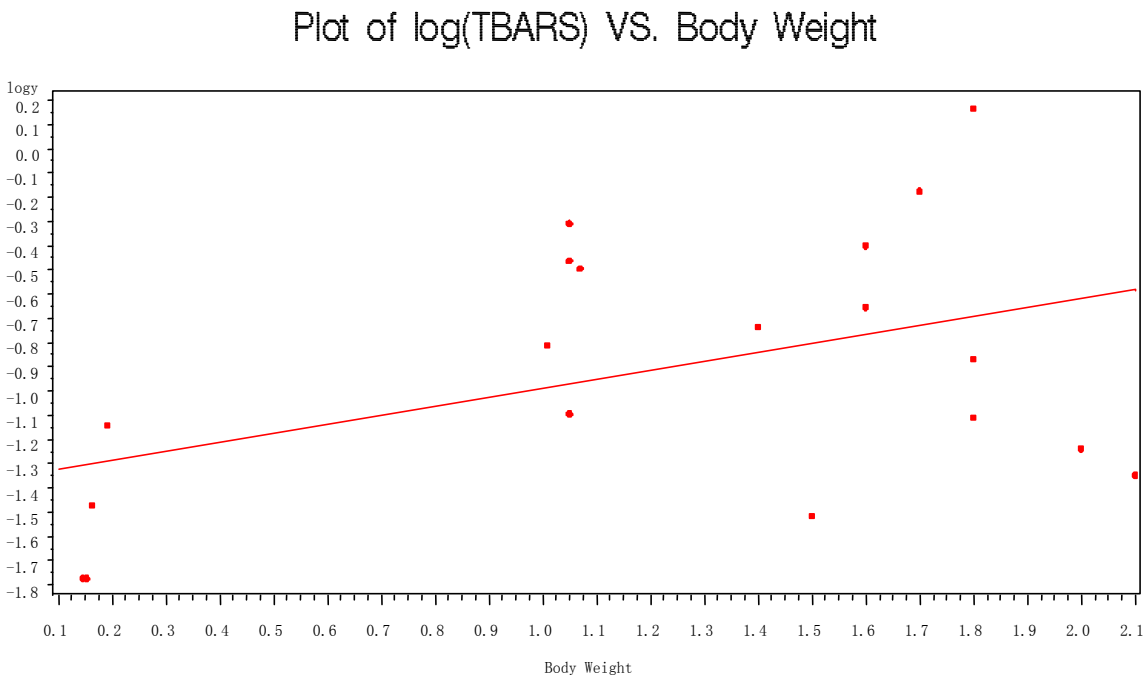
Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = 202.30289x - 7.72922

R^2 = 0.4235, P = 0.0577.

No significant correlation was found between ln(TBARS) and body weight in male budgies.

Appendix 22. Plot of ln(TBARS) and body weight in male guineafowls



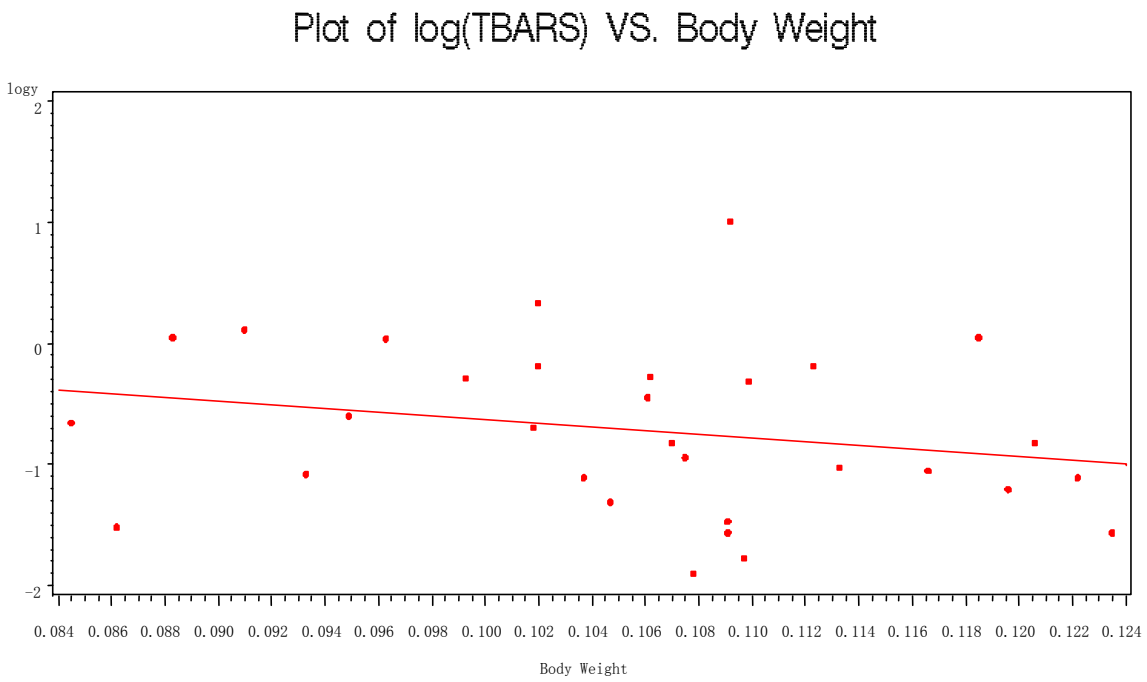
Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = 0.37191x - 1.35873

R^2 = 0.1960, P = 0.0577.

No significant correlation was found between ln(TBARS) and body weight in male guineafowls.

Appendix 23. Plot of ln(TBARS) and body weight in male quails



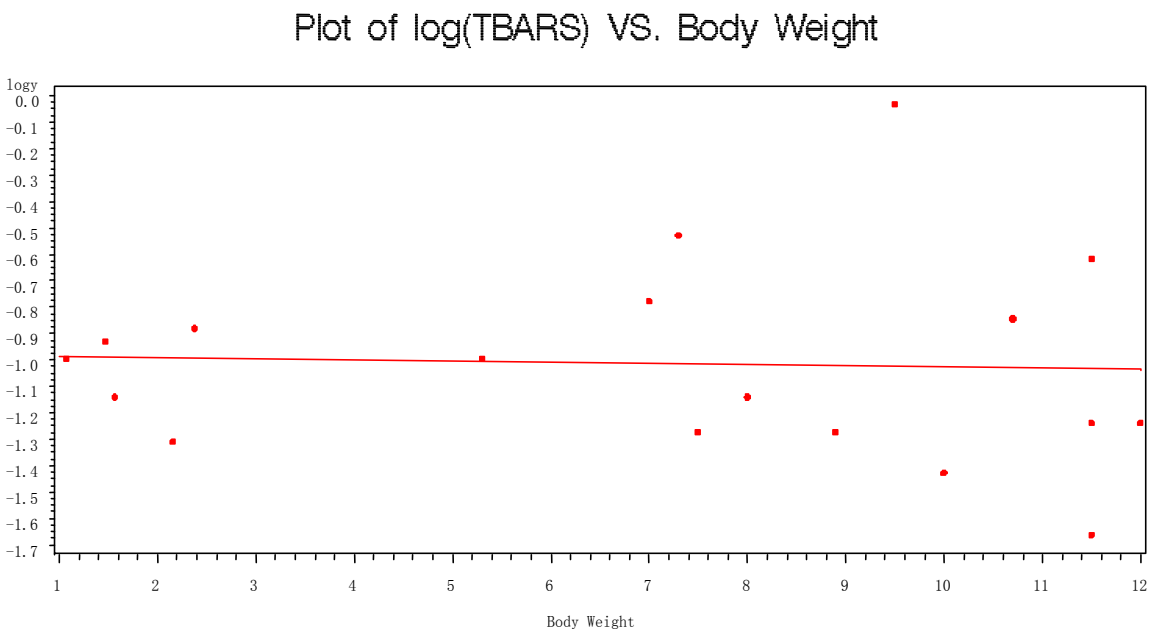
Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = -15.20272x + 0.88782

R^2 = 0.0533, P = 0.2115.

No significant correlation was found between ln(TBARS) and body weight in male quails.

Appendix 24. Plot of ln(TBARS) and body weight in male turkeys



Linear regression model: y is ln-transformed measurement of TBARS, and x is body weight:

y = -0.00430x - 0.98520

R^2 = 0.0020, P = 0.8601.

No significant correlation was found between ln(TBARS) and body weight in male turkeys.

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