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Oxidative Stress in Avian Embyros

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Aging and life span in birds: The role of oxidative damage during development

Trade-offs among structures and functions, and the compromises they require of all organisms, are a central to our understanding of life-history evolution. However, the physiological mechanisms that mediate these trade offs and constrain life history evolution remain largely unknown (Monaghan et al. 2009). The length of the avian embryonic development period varies greatly across species, from 11 to 85 days. Some of this variation can be attributed to differences in embryo size, with larger species typically requiring longer to develop than smaller eggs. This increase in embryo development period as a function of egg size reflects the increased proliferation of cells and tissues needed to produce larger hatchlings (Arendt 1997). The development of larger embryos may also be slowed due to nonlinear scaling of embryo metabolic rates due to changing egg surface-area-to-volume-ratios as egg sizes increase (Rahn and Ar 1974). However, differences in embryonic development due to differences in allometric growth are insufficient to explain the variation among avian development rates (Ricklefs 1998). Ecological explanations for differences in growth rates include variation in predation pressure on eggs (Martin 2007).

Slower development of embryos may have negative consequences. Embryos that develop slowly are more susceptible to sources of time dependent mortality such as nest predation. Studies comparing continental and island populations, where predation pressure is lower due to the lack of common nest predators, suggest that predation pressure selects for a shorter nestling period

(Bosque and Bosque 1995, Schwabl et al. 2007). There is also evidence that females increase the deposition of androgens, which have been correlated with increased growth rates, into embryos in response to high levels of nest predation (Schwabl et al. 2007). Despite this, many tropical avian species that are subject to very high nest predation rates have incubation periods that are long when compared to their temperate counterparts (Martin 2002). Additionally, incubation is costly for the parent(s) as attendance at the nest has been associated with a number of short-term costs including reductions in body mass and in measures of immune function (Coleman and Whittall 1988, Hanssen et al. 2005).

Recent evidence suggests that the rate of embryonic development may be linked to the rate of aging and maximum lifespan. Comparative analyses have found relationships between embryonic development and patterns of aging across species, with decreased longevity in species that develop more rapidly (Ricklefs and Scheuerlein 2001). Intraspecific studies have shown that nutritional stress during early life can profoundly influence several characteristics of adult birds, including reproductive success and annual survivorship (Metcalfe and Monaghan 2003, Barker et al. 1989). Other proposed trade-offs accounting for the relationship between senescence and development period include trade-offs between immune system development and growth rate (Ricklefs 1992). Slower development may allow an organism to produce a more robust immune system that increases its survivorship (Ricklefs 1992). It is also possible that rapid development leads to increased accumulation of cellular

damage due to either an increased rate of damage formation or failure to repair damage, and damage during development may have more profound effects on longevity than damage that occurs later in life. This is often referred to as the High Initial Damage Load (HIDL) hypothesis. Additionally, the HIDL hypothesis suggests that organisms are born with damage incurred during development and therefore that conditions during development may have implications for longevity (Garilov and Garilova 2001). Under this hypothesis, longer living organisms have optimized their development processes to decrease the amount of damage accrued in this period and optimizations may have a trade-off with developmental rate producing the observed correlation between development period and aging.

Oxidative Stress

Oxidative stress has been proposed as a general mediator of life history trade-offs (Monaghan et al. 2009). Aging caused by oxidative damage to DNA and other macromolecules by metabolic processes is a central component of several theories of senescence. Free radicals, molecules with a single unpaired electron in their valence shell, are the primary source of oxidative damage. Studies have indicated that reactive oxygen species (ROS) are involved in a number of biological processes including apoptosis, immune response, cell-to-cell signaling, normal cellular growth and metabolism, as well as cellular senescence (Harman 1957, Finkel and Holbrook 2000). Reactive oxygen species which include the superoxide radical, hydroxyl radical and hydrogen peroxide, can be formed by a number of enzymatic processes where molecular oxygen acts

as a substrate. However, 90% of ROS are formed as a consequence of metabolic activity.

The electron transport chain in the mitochondrion is the source of the majority of intracellular ROS production (Cadenas and Davies 2000). This mainly occurs at two sites within the mitochondrion: NADH dehydrogenase in complex I and ubiquinone-cytochrome c reductase in complex III). At these complexes, electrons are more likely to interact with oxygen to form the free radical superoxide that in turn produces other ROS. The production of ROS by these complexes is primarily a function of metabolic rate, with 0.1% to 4% of molecular oxygen forming ROS (Cadenas and Davies 2000). Once produced, free radicals damage biological macromolecules that then contribute to the senescence-related decline of an organism's function and performance (Finkel and Holbrook 2000). As oxidative damage preferentially causes strand breaks in guanine rich telomeres, ROS can also contribute to shorting of telomeres and accelerated cellular senescence (Von Zgniliki 2002). Absolute telomere length and the rate of telomere attrition has been associated with differences in survival (Heidinger et al. 2012, Barrett et al. 2013). If the production of ROS exceeds an organism's ability to prevent or repair damaged caused by them, the organism is considered to be in a state of oxidative stress.

Organisms have several ways to prevent oxidative stress and reduce the amount of ROS produced by mitochondria and these mechanisms may operate independently of metabolic rate (reviewed in Balaban et al. 2005). In total about 0.2% of all oxygen processed by the mitochondrion forms ROS (Aguilaniu et al.

2003). This amount can be affected by presence of uncoupling proteins that allow dissipation of the proton gradient across the mitochondrial inner membrane as heat rather than coupled with the production of ATP (Finkel et al.2000). In rats (*Rattus rattus*) proton leak associated with uncoupling is responsible for 20-25% of all oxygen consumed and it has been proposed that mitochondrial "inefficiency" is essential to reduce oxidative stress (Brand 2000). Additionally the metabolic substrate can influence the production of ROS by the mitochondrion; the use of fatty acids as a metabolic substrate reduces oxidative stress through an increase in the amount of uncoupling (Hulbert et al. 2007). The composition of mitochondrial membranes also influences the generation of free radicals and varies with species and can change with age (Balaben et al. 2004, Hulbert et al. 2008).

Defenses against oxidative damage

Organisms have developed several different mechanisms of antioxidant defense to counter the production of ROS. Organisms possess both endogenously produced enzymatic antioxidants including superoxide dismutases and non-enzymatic antioxidants such as ascorbate and albumin, both contributing to an organism's total antioxidant capacity (Finkel and Holbrook 2000). Experimental evidence suggests that these enzymatic pathways are essential for proper organismal function. Knockout experiments in mice have indicated high mortality and mitochondrial malfunction in mice unable to produce the enzymatic antioxidant superoxide dismutase 2 (Melov et al. 1999). Additionally, mice that over-expressed catalase (the enzyme that deactivates

hydrogen peroxide, a common ROS) at higher levels lived 20% longer (Schriner et al. 2005).

In addition to mechanisms that limit the extent of oxidative damage, organisms have evolved mechanisms to repair the damage. Multiple methods of DNA repair exist, preserving the integrity of the genetic sequence for proper organism function. There is evidence that an organism's ability to repair declines with age and may contribute to more rapid senescence seen later in life in some organisms (reviewed in Lombard et al. 2005). To date little work has been conducted measuring repair mechanisms in ecological studies exploring life-history trade-offs. Due to mechanisms to prevent and repair damage caused by ROS, a high rate of production of ROS alone does necessarily reflect a state of oxidative stress.

Compared to most vertebrates, birds senesce at a much slower rate and have much longer life spans than other endotherms with similar body sizes (Holmes and Ottinger 2003). This occurs despite their high mass-specific rates of metabolism, given that high rates of metabolic activity should correlate with an equally high rate of ROS which has been linked to the molecular changes associated with aging (reviewed in Holmes and Ottinger 2003). Birds may have evolved more effective defenses against macromolecular damage and/or more effective methods of detecting and repairing damage caused by ROS (Barja 1998). For example, cultures of avian embryonic fibroblasts were more resilient to the exposure to ROS than similar cells derived from mammals (Ogburn et al. 1998). However, there is inconsistent evidence as to whether this increased

resilience was due to an increase in enzymatic antioxidant defenses in birds (Ku and Sohal 1993, Barja et al. 1994). The susceptibility of tissues to oxidative damage may also vary between organisms. In both mammals and birds, species that age more slowly have higher proportions of saturated fatty acids that are more resistant to oxidative stress than unsaturated fatty acids (Hulbert 2008).

The total amount of oxygen consumed by developing embryos of precocial birds increases exponentially as the incubation proceeds peaking at an age that is 80% of the duration of incubation (Vleck and Vleck 1980). Differences in patterns of oxidative stress during development may be influenced by both the rapid increase in oxygen consumption during development as well as changes in the antioxidant defenses and structural properties of embryonic tissues. Yolk lipids, particularly long chain polyunsaturated fatty acyl and antioxidants, are transported at higher total rates into the developing embryo during the latter half of embryo development (Surai 1996). Additionally, the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, can vary both temporally and spatially within developing embryos (Surai et al. 1999). Differences in ROS production, susceptibility to oxidative damage, and antioxidant defenses may result in differences in oxidative stress between tissues within a developing embryo.

Oxidative stress and life history trade-offs

Oxidative stress could mediate the observed relationship between the rate of development and longevity through several different mechanisms. First, there is evidence that increased growth rate produces increased oxidative stress.

Transgenic mice expressing higher levels of growth hormone also grew faster, generated more ROS, had higher indicators of oxidative stress, and had reduced lifespans (Rollo et al. 1996). Oxidative stress has been implicated in accelerated shortening of telomeres, which would result in cells reaching replicative senescence more rapidly (Zglinicki 2002). Oxidative damage to telomeres is not repaired as well as in other areas of the chromosome and therefore increased oxidative stress imposed by rapid growth early in life may have disproportionately large affect on longevity given that most telomere loss occurs early in life (Hall 2004).

Another possibility is that rapid growth prevents investment in the production of robust or redundant antioxidant systems and therefore reduces effectiveness in responding to oxidative challenges. There is evidence that a trade off exists between protein turnover rates and growth rate (Morgan et al. 2000). In an experimental manipulation of growth rates in zebra finches (*Taeniopygia guttata*), accelerated growth was correlated with an increased susceptibility of red blood cells to oxidative stress, a finding that would be consistent with the idea that there may be trade offs between growth rate and mechanisms for self-maintenance (Alonso-Alvarez et al 2007). Oxidative stress has been proposed as a mediator of the relationship between rapid growth and long-term negative effects on performance and survival (Metcalfe and Monaghan 2003, Monaghan et al. 2009).

To date there is little information on the oxidative environment of developing embryos and no information about how oxidative stress changes

over the course of development. To address this, in chapter one I describe patterns of oxidative stress within three tissues from developing Japanese quail (*Coturnix japonica*) sampled at different stages of embryonic development. I found that quail embryos are constantly in a state of oxidative stress and that oxidative damage accumulates in tissues over the course of embryonic development.

If more rapid embryonic growth rates resulted in greater production of ROS without a corresponding increase in antioxidant defense mechanisms, more rapidly developing organisms would have higher levels of oxidative stress than organisms with more prolonged development periods. To explore the relationship between development period and oxidative stress, in my second chapter I compare measures of oxidative stress and antioxidant defenses within embryos from five avian species (domestic chicken (*Gallus gallus domesticus*), Japanese quail (*Coturnix japonica*), mallard duck (*Anas platyrhynchos*), Leach's storm petrel (*Oceonodroma leucorhoa*) and herring gull (*Larus smithsonianus*)) with different embryo development periods. Samples of embryonic tissue from these species were collected at a standardized percentage of their total embryonic development period and then assayed for products of lipid peroxidation, oxidation of DNA and DNA repair capacity. Embryos from species with shorter embryo development periods had higher levels of oxidative stress and did not have any increase in antioxidant defenses. This is consistent with the predictions of the HIDL hypothesis and oxidative stress may mediate the relationship between development period and lifespan.

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Increasing lipid peroxidation in during development in Japanese quail(*Coturnix japonica*) embryos

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Abstract

The growth of the avian embryo is paralleled by an exponential increase in the rate of whole- embryonic oxygen consumption, which potentially increases oxidative damage. We characterized age-related patterns of tissue lipid peroxidation in brain, liver and heart tissue of developing Japanese quail (*Coturnix japonica*) embryos between nine and 15 days of age, over which embryo mass increased by a factor of six. Lipid peroxidation was quantified in each tissue by spectrophotometric measurement of malondialdehyde by the thiobarbituric acid reactive substances assay. In all tissues, lipid peroxidation increased greatly as development proceeded. Increases in the levels of malondialdehyde were strongly related to the amount of oxygen consumed by the developing embryo consistent with the hypothesis that oxidative stress results from the production of free radicals due to oxidative metabolism.

Introduction

As avian embryos develop, the rapid increase in embryo mass is coupled with a corresponding increase in metabolic rate (Vleck and Vleck 1980), quantified for a variety of species (e.g., Hoyt 1987, Vleck and Vleck 1987). Oxidative metabolism accounts for 90% of the reactive oxygen species (ROS) produced by organisms (Finkel and Holbrook 2000, Balaban et al. 2005). Although individuals possess mechanisms such as antioxidant defenses and repair mechanisms to limit damage caused by ROS production, little is known about the accumulation of oxidative stress during embryonic development, which might impact growth, survival, and reproductive success in the chick and the adult (Monaghan et al. 2009)

Oxidative stress results from the imbalance between the production of ROS by oxidative metabolic processes and an organism's ability to prevent or repair damage caused by their presence in tissues. In chicken embryos, antioxidant defenses vary temporally and between tissues, and tissues and tissues differ in their susceptibly to ROS induced damage due to differences in lipid composition (Surai 2003). Within avian eggs, the largest sources of antioxidants are those that are maternally deposited in the yolk (Surai 2003). Over the course of embryonic development, yolk antioxidants are transferred to the developing embryo and partitioned differentially between tissue types (Surai and Sparks 2001). Most of the yolk antioxidants are stored in the developing

liver, giving the liver higher levels of non-enzymatic antioxidants than other tissues during development (Maldjian et al. 1996, Surai et al. 1996)

While laying females actively transport antioxidants into yolks, they may face a trade off between the deposition of antioxidants into eggs and the maintenance of the maternal antioxidant stock (Rubolini et al. 2006). Experimental supplementation of antioxidants through both direct injections of antioxidants into eggs and supplementing antioxidants to laying females increased the hatchability of eggs in addition to improving growth and immune responses in newly hatched chicks (Lin et al. 2004). This suggests that antioxidants are both important to normal development and potentially limited.

The rate of oxygen consumption by developing embryos of precocial birds increases exponentially as incubation proceeds peaking at an age that is 80% of the duration of incubation (Vleck and Vleck 1980). Differences in patterns of oxidative stress during development may be influenced by both the rapid increase in oxygen consumption in development as well as changes in the antioxidant defenses and structural properties of embryonic tissues. Yolk lipids, particularly long chain polyunsaturated fatty acyl and antioxidants are transported at higher total rates into the developing embryo during the latter half of embryo development (Surai 1996). Additionally, the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase can vary both temporally and spatially within developing embryos(Surai 2003).

In this study we characterize patterns of lipid peroxidation, indicative of oxidative damage in three tissues across embryonic development in Japanese

quail. While work describing patterns of antioxidant defenses in developing embryos exists, there has been comparatively little information on the *in vivo* patterns of oxidative stress.

Materials and Methods

Animals

We measured levels of lipid peroxidation in Japanese quail (*Coturnix japonica*) embryos obtained as fertilized eggs through a commercial supplier (eFowl, Middle River MN) and incubated in a Hova-Bator incubator (Model1620N, G.Q.F. Manufacturing Company Inc. Savannah GA) at the University of Missouri-St. Louis. Embryos were incubated at 37.5° and 65% humidity. The incubator was equipped with an automatic egg turner simulating development conditions during natural incubation. Temperature and humidity were checked twice a day.

Sample Collection

Japanese quail incubation length is typically 17 days. Quail embryos were sampled randomly on days 9 (n=4), 11 (n=3), 13 (n=3) and 15 (n=4) after the start of incubation. At each time point, samples of 0.1-0.5g brain, liver and heart tissue were placed in physiological buffered saline (PBS) with 10% DMSO (Sigma, St. Louis MO) and stored at -80°C until analyzed. Samples from all tissues were thawed and homogenized at 100mg/ml in ice-cold PBS using a Kontes dounce-type glass homogenizer (Kimble-Chase, Vineyard, NJ). Following homogenization, samples were treated with 1% butylated hydroxytoluene (BHT) to prevent further oxidation.

Lipid Analysis

Lipid peroxidation caused by ROS results in the formation of several end products including malondialdehyde. Malondialdyde was assayed in the tissue samples spectrophotometrically by measuring levels of thiobarbituric acid reacting substances (TBARS). The damage caused by ROS to lipids causes the formation of malondialdehyde which reacts with thiobarbituric acid under acidic conditions to form a chromogenic product that can be measured spectrophotometrically (Okhawa et al. 1979). The TBARS assay was performed on 200μl of the sample using the Oxiselect TBARS Assay Kit (CellBio Labs, San Diego, CA). TBARS products were extracted using n-butanol to prevent contamination and interference by hemoglobin. The butanol layer was transferred to a 96 well plate and the absorbance (lambda =532) was measured on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Duplicate values were averaged to estimate the TBARS concentration of each sample.

Statistical Analyses

Data were analyzed by means of analysis of variance (ANOVA) and linear regressions carried out using R software version 2.8.1(R-Core Team 2012). Metabolic rates of Japanese quail embryos were obtained from Vleck and Vleck (1980). Data from graphs in Vleck and Vleck (1980) summarizing patterns of embryonic metabolic rate within precocial species were used to estimate mass specific metabolic rates at each sampling point that may be correlated with the intensity of potential ROS production. These rates were used to calculate the

percent of the accumulated total embryonic oxygen consumption over the entire development period at each sampling date.

Results

Concentrations of malondialdehyde within homogenates of embryonic tissues increased five- to nine-fold between days nine and 15 of embryo development (Table 1). The magnitudes of increase were not the same for all tissues, with brain having the highest increase in levels of malondialdehyde and heart the lowest (ANOVA, $F=14.35$, $df=2,36$, $p<.001$). We found no relationship between the amount of malondialdehyde and the mass-specific metabolic rate of the embryo at the time of sampling (linear regression, $p = 0.16$, $R^2 = 0.54$; figure 1). When analyzed by multiple regressions including day of incubation, mass specific metabolic rate, and percentage of cumulative oxygen consumption, levels of malondialdehyde were only significantly related only to cumulative embryonic oxygen consumption (Table 2. $F=95.15$, $df=3,38$, $p<0.001$, $R^2= 0.88$; figure 2).

Discussion

Across all tissues types, there was an increase in the amount of lipid peroxidation in tissues of developing quail embryos as measured by the TBARS assay. This result implies that as development proceeds, the antioxidant defense systems within embryos cannot keep up with the increase in oxygen metabolism. The magnitude of the increase in lipid peroxidation varied between tissues with brain homogenates having the largest increase in lipid peroxidation.

This could be due to the increasing accumulation of unsaturated fatty acids in neural tissue over the course of development (Surai 2003).

In our analysis of the relationship of malondialdehyde to total oxygen consumption, age of the embryo and metabolic rate, total accumulated oxygen consumption was the best predictor of the amount of malondialdehyde for all tissues. The lack of correspondence between metabolic rate and levels of malondialdehyde suggests that the antioxidant systems of developing tissues slowly accumulate oxidative damage throughout incubation. Our observation that levels of malondialdehyde increase in proportion to total amount of oxygen consumed is consistent with the notion that production of ROS is tightly coupled to oxygen consumption, with about 90% of all ROS produced as a byproduct of metabolic processes (Balaban et al. 2005). During embryonic development, the rate at which unsaturated lipids are transferred from the yolk into the developing embryo has been show to increase greatly (Surai 1999). This could help account for the observed patterns of malondialdehyde accumulation.

Less oxidative damage that occurs during development than post-hatching in the chicken (*Gallus gallus domesticus*) due to the transition to direct atmospheric respiration (Surai 2003). For example, Balogh et al. (2001) demonstrated that in newly hatched chickens, the levels of lipid peroxidation were two- to three-fold higher than in our samples from 15 day old embryos, and ultimately were twice as high on day 21 of postnatal growth than at day of hatching. The transition from chorioallantoic to pulmonary respiration is accompanied by an increase in the rate of oxygen metabolism by 60% (Surai

2003). Following hatching, the stores of carotenoid antioxidants are rapidly distributed to tissues from the liver. In contrast to the liver, the developing brain has low levels of most major micromolecular antioxidants, with the exception of ascorbic acid (Surai et al. 1999). In brain tissue, the amount of lipid oxidation continues to increase in chickens up until at least the third week of life, which corresponds to the typical window of onset for nutritional encephalomalacia which is associated with deficiency in the antioxidant vitamin E (Balogh et al. 2000). When challenged by radical-generating free iron ions, lipids in the brain were found to be much more susceptible than those present in the livers of newly hatched chicks (Surai et al. 1999). If antioxidant defenses in brain tissue and liver tissue of our Japanese quail are similar to those in developing chicken embryos, this would imply that lower levels of ROS are produced in the brain when compared to the liver as they did not differ significantly in the total amount of lipid peroxidation.

Despite the predictions of the free radical theory of aging that antioxidant defenses should be strongest early in life, immature and juvenile birds often have lower levels of antioxidant defenses (Harman 1956, Blount et al. 2003). Recent work suggests that oxidative stress during early life may have effects that last into adulthood (Haussmann et al. 2012, Heidinger et al. 2012). For example, in captive populations of greater flamingos (*Phoenicopterus ruber roseus*), the relationship between resistance to oxidative stress and age was convex, with the middle-aged flamingos being more resistant to oxidative stress than both their younger and older conspecifics (Devevey et al 2010). There is also evidence that

an adult bird's susceptibility to oxidative stress can result from alterations in nestling growth rate and suggests that bird's natal environment can influence oxidative stress at later life-history stages (Alonso-Alvarez et al. 2006). More research is needed to clarify how vulnerability to oxidative stress changes over an organism's life.

Oxidative stress appears to have diverse effects on the development of vertebrates (reviewed in Dennery 2007) and is increasingly thought of as being a potential mediator of life-history trade-offs (Monaghan et al. 2009). ROS play important roles in development processes by influencing cellular redox states that regulate transcription factors affecting both differentiation and apoptosis (Hitchler and Domann 2007). While essential for normal developmental, a shift to higher levels of oxidative stress can have negative consequences for embryonic development and in some cases adult phenotype (Thomson and Al-Hasan 2012). Elevated levels of oxidative stress have been demonstrated to impair cardiovascular health and depress neonatal size at birth of rats (*Rattus rattus*) (Cambonie et al. 2007). Tissues may be more resilient or tolerant to the effects of oxidative stress in later stages of embryonic development where we found the highest levels of oxidative stress, than earlier in development.

Conditions during development may also influence oxidative stress post-hatching. While chicken embryos that were experimentally exposed to higher levels of testosterone during embryonic development did not have higher levels of oxidative stress, they had reduced capacity to mitigate the effects of an oxidative challenge following hatching (Treidel et al. 2013). In contrast,

experimental increases in corticosterone levels during development increased levels of oxidative stress (Haussmann et al. 2012). Both of these hormones can be deposited maternally into the yolk of eggs and alterations to the oxidative environment in developing chicks may therefore be mediated by oxidative stress.

Our results suggest that during embryonic development, ROS production and antioxidant defenses are imbalanced, leading to the accumulation of oxidized lipids and presumably other products of oxidative stress. The accumulation of products of lipid peroxidation during development was directly related to the total amount of oxygen consumed by the embryo. While there are negative costs associated with increased levels of oxidative stress during development, Japanese quail are consistently in a state of oxidative stress during embryonic development. An important step for research in this area would be to document the generality of this pattern and the consequences of oxidative damage for post-hatching development and function.

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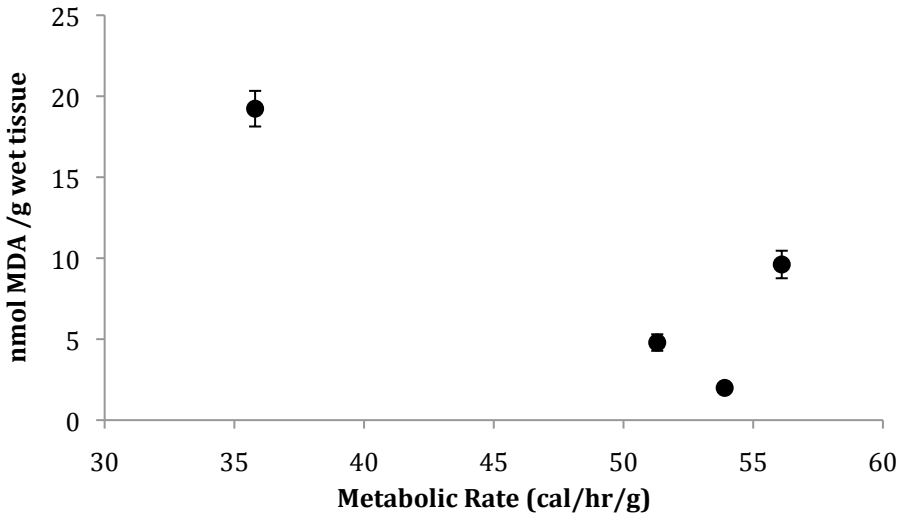
Figure Legends

Figure 1: Relationship between the amount of malondialdehyde measured in samples of brain tissue and estimates of the metabolic rate of the developing embryo. ± Corresponds to the standard error of the mean. Patterns in other tissues are similar

Figure 2: Relationship between amount of malondialdehyde measured in samples of brain tissue and percentage of total oxygen consumed by a developing quail embryo. ± Corresponds to the standard error of the mean. Patterns in other tissues are similar


25
20
15
10
5
0
nmol MDA /g wet tissue
30
35
40
45
50
55
60
Metabolic Rate (cal/hr/g)

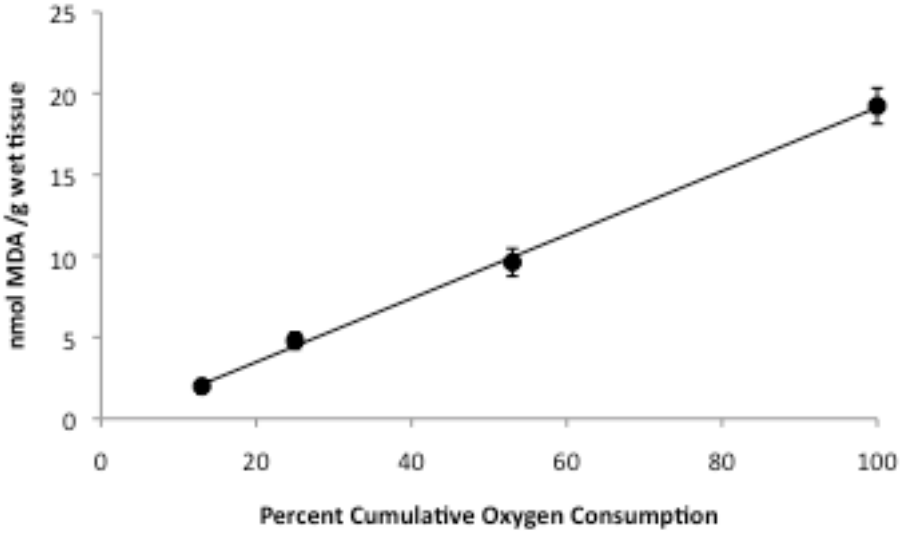

25
20
15
10
5
0
nmol MDA /g wet tissue
0
20
40
60
80
100
Percent Cumulative Oxygen Consumption

Table 1: Malondialdehyde values represent nmol/g wet tissue at each sampling. ± Corresponds to standard error. Estimated metabolic rates are expressed as cal/hr/g wet yolk-free embryo mass.

Age in days	Malondialdehyde			Estimated Metabolic Rate
	Brain	Liver	Heart	
9	1.99 ± 0.35	1.70±0.27	1.65 ± 0.23	67.38
11	4.79 ± 0.27	3.59 ± 0.51	2.81 ± 0.17	56.1
13	9.61 ± 1.07	7.77 ± 0.85	5.82 ± 0.50	51.3
15	19.23 ± 0.85	15.85 ± 1.10	8.83 ± 1.02	35.81

Table 2: Predictors of lipid peroxidation analyzed through multiple regression.

Independent Variables	β	t	p
Day of Incubation	0.29	1.09	0.87
Percent cumulative oxygen consumption	0.42	2.58	0.013
Mass specific metabolic rate	0.01	0.08	0.94
Multiple R^2= 0.782, F(3,38)= 45.43	p < 0.001		

Less oxidative stress in embryonic tissues of species with longer development periods

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Summary

Oxidative stress has been implicated in mediating trade-offs in the evolution of life histories. Higher metabolic rates associated with rapid growth and shorter development periods may increase oxidative stress and accumulated cellular damage in embryonic tissues. We investigated damage to DNA and lipids, as well as the efficacy of DNA repair in erythrocytes of developing embryos of five avian species with different embryo development periods. Samples were collected from late-stage embryos of five species: chicken (*Gallus gallus domesticus*), Japanese quail (*Coturnix japonica*), herring gull (*Larus smithsonianus*), mallard duck (*Anas platyrhynchos*) and Leach's storm petrel (*Oceonodroma leucorhoa*). Incubation in these species ranges from 17 days (quail) to 42 days (storm petrel). We measured differences in levels of oxidative damage to lipids using the thiobarbituric reactive substances assay and found that the levels of oxidative damage in brain, liver and heart were lower in

species with longer embryonic development periods. Using single cell gel electrophoresis we also found a similar pattern in oxidative damage to DNA, with lower levels in species with more prolonged embryonic development periods. However, we found no difference between species or tissues in non-enzymatic antioxidants or in the repair of an oxidative challenge to DNA. Collectively, these results suggest that increased levels of oxidative stress may be a consequence of more rapid embryonic development.

Keywords

Oxidative stress, development, antioxidants, avian embryo

Introduction

Stress and damage suffered during embryo development can influence the physiology, reproductive success and survival of individuals (Lindenström 1999). The length of the avian embryonic development period varies greatly across species, and several explanations for this variation have been proposed. Some of this variation can be attributed to differences in embryo size, with larger eggs taking longer to hatch than smaller eggs. The longer duration of the embryo development period in larger species with larger eggs results from the increased time required for proliferation of the larger number of cells in larger hatchlings (Arendt 1997). The slower development of larger embryos may also be related to nonlinear scaling of embryo metabolic rates in response to changing egg surface-area-to-volume-ratios as egg sizes increase (Rahn and Ar 1974). However, differences in embryonic development due to differences in

allometric growth are insufficient to explain the variation among avian development rates (Ricklefs 1993).

Longer incubation periods are associated with a number of negative consequences. Embryos that develop for a longer period are likely subject to prolonged susceptibility to sources of time-dependent nest mortality, including predation. Comparisons of postnatal growth rates in continental and island populations, where islands have fewer common nest predators, suggest that predation pressure selects for a shorter nestling period (Bosque and Bosque 1995, Schwabl et al. 2007) and more rapid nestling growth and development (Remes and Martin 2002). Despite clear advantages of rapid development, incubation periods vary independently of egg size among species with similar functional capacity at hatching indicating that variation in embryo growth rate and factors favoring the slower development of embryos of some species remain unclear (Ricklefs 1993, Dimitriew 2010).

Comparative analyses have found relationships between embryonic development and patterns of aging across species, with decreased longevity in species that develop more rapidly (Ricklefs 1993; Ricklefs 2010a). One consequence of more rapid embryo development may be increased accumulation of cellular damage due to either an increased rate of damage formation or failure to repair damage. This damage early in life when organisms are growing rapidly may have disproportionately large effects on longevity compared to damage that occurs later in life (Gavrilov and Gavrilova 2001, Mangel and Munch 2005). This is often referred to as the High Initial Damage

Load (HIDL) hypothesis (Gavrilov and Gavrilova 2004). This hypothesis suggests that organisms are born with damage accrued during development and that developmental strategies that reduce the amount of damage during development may extend longevity (Gavrilov and Gavrilova 2004). Under this hypothesis, organisms with longer potential life spans should have optimized their development processes to minimize the damage accrued during this period.

Oxidative stress and antioxidant defenses have been implicated in mediating trade-offs in evolutionary ecology (Monaghan et al. 2009, Constantini 2008). Reactive oxygen species (ROS) capable of producing oxidative damage are generated by intracellular metabolism and therefore are associated with metabolic rates (Finkel and Holbrook 2000). Oxidative stress results from differences between the rate of free radical production and detoxification. The ubiquity of the challenge presented by ROS has driven the evolution of a number of mechanisms to prevent and repair damage caused by ROS (Barja et al. 2004). Higher levels of oxidative stress might result from faster development if the increased metabolic demands of development and ROS production are not associated with equivalent investment in antioxidant defense systems and repair. A similar pattern would be found if rapid growth resulted in a diversion of limited resources away from prevention and repair of oxidative stress (Kim et al. 2011).

To explore the role that oxidative stress may play as a potential cost of more rapid embryo development, we measured levels of oxidative stress during

development by quantifying markers of lipid peroxidation and the oxidation of DNA in five species of birds with differing embryo development periods. Differences in oxidative stress both across species and within individuals can depend on differences in the underlying protection of tissues to oxidative stress and therefore we measured differences in both the antioxidant capacity and the repair of oxidative damage to DNA caused by an H_20_2 challenge. If a tradeoff existed between the rate of embryonic growth and oxidative stress we would expect to see lower levels of DNA oxidation in embryos of species with shorter incubation periods. We also would expect longer-lived organisms to invest more in self-maintenance and to possess more robust systems for mitigating oxidative stress, leading to lower levels of damage when challenged with H_20_2 and higher levels of antioxidants.

Materials and Methods

Bird Sampling

We measured levels of lipid peroxidation and antioxidant status in embryos of domestic chicken (*Gallus gallus domesticus*, n=19), Japanese quail (*Coturnix japonica,* n=17), mallard duck (*Anas platyrhynchos*, n=14), Leach's storm petrel (*Oceonodroma leucorhoa*, n= 13) and herring gull (*Larus smithsonianus*, n=13). Fertilized duck, quail and chicken eggs were ordered from a commercial supplier (eFowl, Middle River MN) and incubated in a Hova-Bator {Model1620N} incubator (G.Q.F. Manufacturing Company Inc. Savannah GA) at the University of Missouri-St. Louis. Herring Gull and Leach's storm petrel embryos were

collected from Kent Island, New Brunswick, Canada in July 2010. All embryos were sacrificed at 70% of the time through the total incubation period. Samples of 0.1-0.5g of brain, liver and heart tissue were placed in physiological buffered saline (PBS) with 10% DMSO (Sigma St. Louis MO). A small (<10μl) blood sample was taken from each embryo and placed in ice-cold buffer containing 90% newborn bovine serum and 10% DMSO (Sigma-Aldrich, St. Louis, MO). All samples were immediately frozen in the gas phase of liquid nitrogen and stored at -80°C until analyzed.

Lipid Analysis

Lipid peroxidation caused by ROS results in the formation of several end products including malondialdehyde. Malondialdehyde was assayed in the tissue samples spectrophotometrically by measuring levels of thiobarbituric acid reacting substances (TBARS). The damage caused by ROS to lipids causes the formation of malondialdehyde that reacts with thiobarbituric acid under acidic conditions to form a chromogenic product that can be measured spectrophotometrically (Okhawa et al. 1979). The TBARS assay was performed on 200μl of the sample using the Oxiselect TBARS Assay Kit from CellBio Labs (San Diego, CA). TBARS products were extracted using n-butanol to prevent contamination and interference by hemoglobin. The butanol layer was transferred to a 96 well plate and the absorbance (lambda =532nm) was measured on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Duplicate values were averaged to calculate the TBARS concentration of each

sample. Total lipids were extracted using a standard methanol/chloroform extraction and quantified by mass.

Single cell gel electrophoresis

Single cell gel electrophoresis (SCGE) (Collins 2009) was performed on erythrocytes after thawing in a 37° C water bath. Cells were suspended in 0.7% low melting point agarose in PBS (pH 7.4) and then placed on pre-scored slides below a cover slip. After the agarose had solidified, the cover slips were removed and cells were allowed to lyse for one hour using a 1% Triton-X solution (2.5M NaCL, 0.1M EDTA acid, 10mM Tris, pH 10). Slides were then washed three times for five minutes with cold enzyme reaction solution (40mM HEPES, 0.1M KCl, 0.5mM EDTA, 0.2mg/ml BSA, pH 8.0). Following the final wash, 50µl of the enzyme reaction buffer containing the enzyme endonuclease III, a restriction enzyme that cleaves strands of DNA at oxidized pyrimidines (New England Biolabs, Ipswich MA) were placed onto the gel and allowed to incubate for 45 minutes. Slides were then immersed in an alkaline electrophoresis solution (0.3M NaOH, 1mM EDTA) for 40 min prior to 30 minutes of electrophoresis at 25 V. Upon completion of electrophoresis, slides were washed three times for five minutes in a neutralizing solution (0.4M Tris, pH 7.5). Slides were then stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO) and analyzed through fluorescence microscopy on Zeiss Axioscope. Each slide was assigned a number and scored blindly. One hundred nucleoids were scored per slide on a scale ranging from zero

(undamaged, no tail) to four (all DNA in tail) to produce a final comet score per slide out of 400.

H_2O_2 Challenge

To measure the repair of DNA damage caused by ROS, embedded cells were exposed to 50 μl of 1mM H_20_2 and incubated for 5 minutes at 4˚C. Slides were then briefly rinsed in PBS. One slide per individual was then placed immediately in the lysis solution while a corresponding slide was allowed to incubate at 37˚C for thirty minutes. Nucleoids on both slides were scored as detailed above. Repair capacity is reported as the difference in comet score between the two slides.

Antioxidant Capacity

The Trolox equivalent antioxidant capacity (TEAC) was performed using an antioxidant assay kit (Cayman Chemical, Ann Arbor, MI, USA). PBS-diluted homogenates were treated with the chromogenic peroxidase metmyoglobin 2,2-azinodi-[3-ethylbenzthiazoline sulphonate] (ABTS). The hydrogen peroxide reacts with the ABTS to form a radical cation, which absorbs ultraviolet light at 260 nm. Some of this radical production is prevented by the presence of antioxidants within the sample and absorbance values derived from tissue were compared to a standard curve of 1 mM Trolox (Miller et al. 1993, Shea et al. 1997). Samples were analyzed on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

Metabolic Intensity

To evaluate the role that differences in embryo metabolic rates have on oxidative damage during development, data on whole egg metabolic rates summarized in Vleck et al. (1980) were incorporated into our analyses. Data on whole embryo metabolic rates were used to calculate an index of metabolic intensity (total kcals of embryonic metabolism)/(g hatch weight × day^{-1}) for each species. This value represents the relative rate of oxygen consumption by a developing embryo per unit mass, that is, relative metabolic intensity.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) and linear regressions carried out in R software version 2.8.1(R-Core Team 2012). Metabolic rates of Japanese quail embryos were obtained from Vleck and Vleck (1980).

Results

Lipid peroxidation was significantly higher in liver tissue than heart and brain, which did not differ from each other (F-value= 8.1**,** df=2 $P<0.001$) as well as among species (F-value= 32.9, df= 4, $P<0.001$). Levels of lipid peroxidation were negatively related to incubation length in cardiac tissue ($p=0.025$, $r^2=0.82$, $n=5$), but the similar trends in brain ($p=0.26$, $r^2= 0.39$) and liver ($p=0.38$, $r^2= 0.16$) were not significant (Fig. 1). An analysis of variance including incubation length, tissue type, and metabolic intensity revealed that levels of malondialdehyde were related to both incubation length (F-value = 33.5 df=1, $p <0.001$) and tissue type (F-value=4.7, df=2, $p=0.01$) were related to levels of malondialdehyde. In all cases, levels of malondialdehyde were lower in tissues of embryos of the

more slowly developing species. A similar pattern existed between lipid peroxidation and longevity, however this relationship was not significant (heart: $p=0.07, r^2=0.61$; brain: $p=0.30$, $r^2= 0.13$; liver: $p=0.39$, $r^2= 0.02$). Antioxidant status and incubation length also were unrelated in heart ($R^2= 0.13$, $p=0.90$) and brain tissue ($R^2= 0.52$, $p=0.13$). Trolox assays of liver samples were uninterpretable and were excluded from analyses. There was no relationship between whole-embryo metabolic intensity and lipid peroxidation in any of the tissues we analyzed, either through regression analysis (brain $p=0.18$, $r^2= 0.27$; heart $p=0.186$, $r^2=0.32$; liver $p=0.27$, $r^2= 0.17$) or by ANCOVA (F-value= 1.30, df =1, $p=0.25$).

Overall levels of oxidative damage in avian embryo erythrocytes measured by SCGE were low with little migration of DNA away from the nucleoid. Nonetheless, the mean comet score was negatively related to incubation length (Fig 2. $P=0.02$, $r^2=0.87$) and a similar relationship was found between mean comet score and maximum lifespan ($p=0.02$, $r^2=0.83$). Mean comet score was not related to whole-embryo metabolic intensity or to metabolic rate at the time of sampling. The H_20_2 slides that were run immediately and not given time to repair the induced damage exhibited significantly higher comet scores than those allowed to repair DNA damage for 30 minutes($t=12.41$, $p<0.01$). However, DNA repair following an oxidative challenge was not related to incubation length: ($r^2=0.04$, $p=0.74$), maximum lifespan ($r^2=0.060$, $p=0.68$) or metabolic intensity ($r^2<0.01$, $p=0.49$).

Discussion

We measured levels of oxidative stress in four tissues from embryos of five different species spanning a range of incubation lengths. For all tissues, we found a pattern where embryos from species with shorter embryo development periods had higher levels of lipid peroxidation than those with longer development periods. The relationship with incubation length was strongest for oxidative damage to DNA. Oxidative damage to the DNA of erythrocytes as measured by SCGE was low with most slides exhibiting high proportions of intact nucleoids. While we cannot directly compare our measurements of lipid peroxidation and oxidative damage to DNA, DNA appears to be less susceptible to the effects of oxidative stress during development.

Our results suggest that despite potential ecological risks associated with prolonged incubation, slow embryonic growth may confer physiological advantages that due to the relationship between incubation length and longevity may influence organismal performance and survival at later life history stages. Differences in our measures of oxidative stress are unlikely to be due to micromolecular antioxidants as we observed no differences between species in antioxidant levels in any of the tissues collected. Nor did we observe no differences between species in the amount of DNA that was repaired in experimentally damaged cells.

Lipid peroxidation differed between tissues, with brain lipids having the lowest levels relative to other tissues for all species. Oxidative stress occurs when the generation of reactive oxygen species exceeds an organism's

antioxidant defenses. Although we could not measure levels of antioxidants in liver tissue reliably, the high level of peroxidation is not likely due to low antioxidant levels. Over the course of development, yolk carotenoids are sequestered by the liver and remain there until hatching (Surai et al. 1996). The brain exhibited the lowest level of oxidative stress despite having the highest proportion of unsaturated lipids, which are readily oxidized (Surai et al. 1996).

Our measurements of antioxidant status and repair of oxidative damage did not explain the interspecific differences in oxidative stress suggesting that differences in the amount of oxidative stress may depend on differences in free radical production. Although our index of metabolic intensity did not explain the between-species differences in oxidative stress, it may not accurately correspond to the amount of ROS produced as differences in the production of ROS have been observed between long-lived and shorter-lived species with similar metabolic rates (Robert et al. 2007). While we did not detect differences in antioxidants within embryonic tissues, some evidence indicates that maternal deposition of carotenoids is higher in species that have higher embryonic growth rates (Deeming and Pike 2013). The absence of differences in the amount of antioxidant levels measured across species might be related to differing rates of their depletion in embryos of different species corresponding to our measured differences in oxidative stress. In this study we did not measure differences between activity of antioxidant enzymes or tissue specific metabolic rates that might alter the generation of ROS or prevention of oxidative stress.

Our results are consistent with the hypothesis that oxidative stress may mediate the trade-off between the rate of organismal development and longevity, but how oxidative stress ultimately influences an individual's long-term survival is unclear. The potential importance of oxidative stress during early development is supported by a cohort study of zebra finches, in which the lengths of telomeres (the repetitive DNA sequences that cap the end of eukaryotic chromosomes) were measured multiple times over the life span, and which found that the best predictor of longevity was telomere length measured at 25 days after hatching (Heidinger et al. 2012). Within vertebrates, the majority of telomere attrition occurs early in life (Brummendorf et al. 2002; Hall et al. 2004). While Heidinger et al. (2012) did not measure oxidative stress directly, telomeres are readily oxidized, and the observed variation in telomere length early in life may be due to differences in oxidative stress (Aviv et al. 2003; Bakaysa et al. 2007).

Our results indicate that oxidative stress during early development, which is directly related to embryo growth rate, potentially constrains organismal longevity through a trade-off between growth rate and oxidative stress. This relationship may drive the strong correlation between embryonic development and longevity among avian taxa. The physiological mechanisms responsible for the differences in levels of oxidative stress among different tissues as well as between species remain unclear. Future work exploring differences in the rate of free radical production, or in aspects of the antioxidant

defense systems in these species may clarify the observed relationship between incubation length and oxidative stress.

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Table 1. Avian species studied, incubation length, maximum lifespan and metabolic intensity

species	incubation length (days)	maximum lifespan (years)	metabolic intensity (cal/g/day)
Japanese quail	16	6	21.3
chicken	21	15	16.9
mallard duck	25-26	29	18.4
herring gull	26-27	30	22.8
Leach's storm petrel	42	36	25.6

Table 2. Results of linear regression models between species life-history characteristics and tissue levels of malondialdehyde (n=5).

	R^2	p
Incubation Length x Lifespan	0.89	0.01
Lifespan x Metabolic Intensity	0.01	0.852
Incubation Length x Liver MDA	0.16	0.38
Incubation Length x Brain MDA	0.26	0.39
Incubation Length x Heart MDA	0.82	0.03

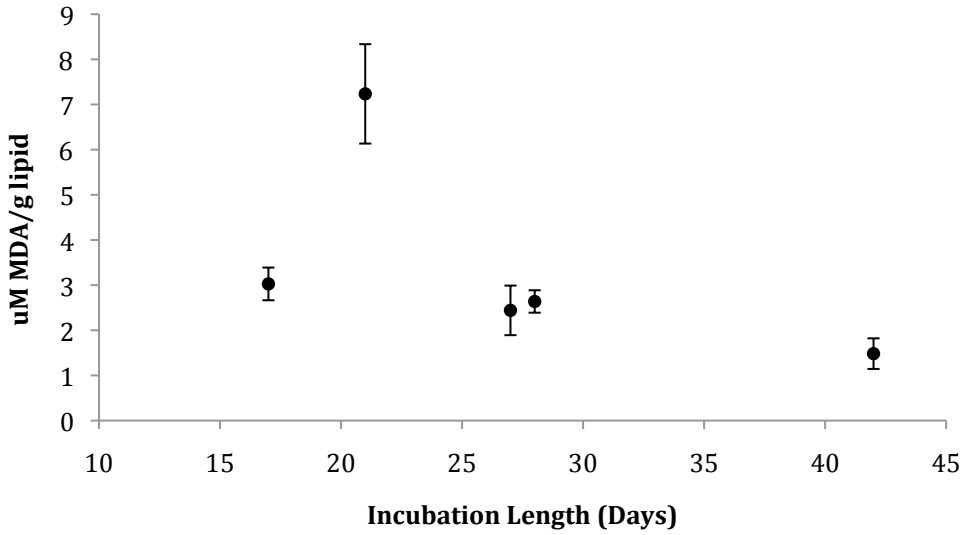
Figure legends:

Figure 1. Lipid peroxidation as a function of incubation length. A) brain ($p=0.262$, $r^2= 0.387$); B); heart ($p=0.025$, $r^2=0.806$); C)liver ($p=0.378$,$r^2= 0.156$). Results for lipid peroxidation with respect to maximum lifespan presented similar results.

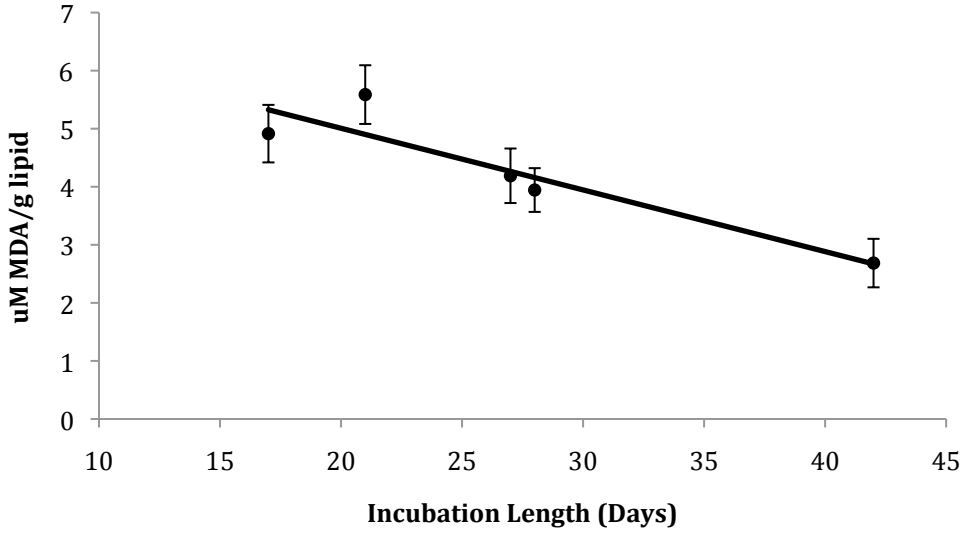
Figure 2. Comet Score as a function of incubation length ($p=0.02$, $r^2=0.83$). Results for comet scores with respect to maximum lifespan presented similar results. Error bars represent the standard error of the mean

Figure 3. Mean comet scores from the H_20_2 challenge. Error bars correspond to the standard error of the mean. HERG, Herring gull; LESS, Leach's storm-petrel; JAQU, Japanese quail; MALL, Mallard duck; CHICK, chicken.

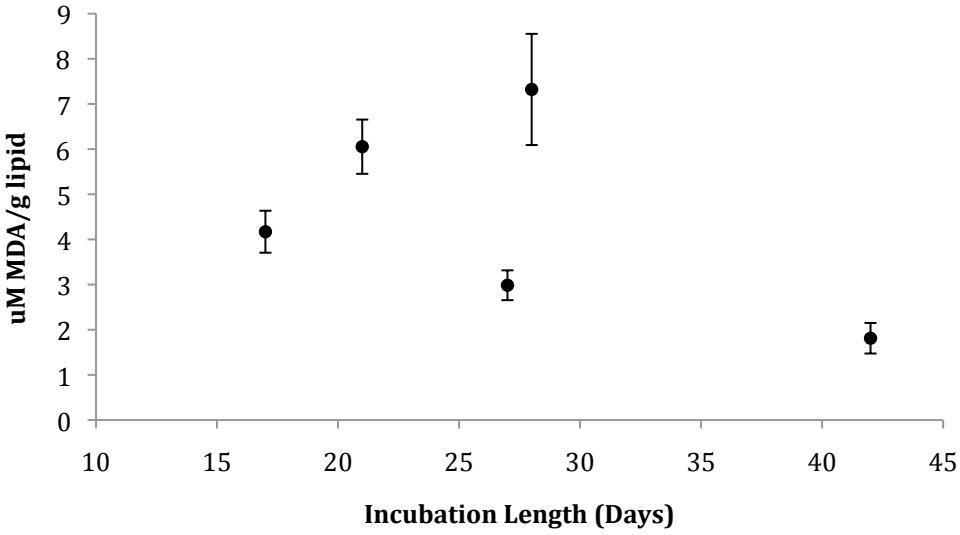
A) Brain

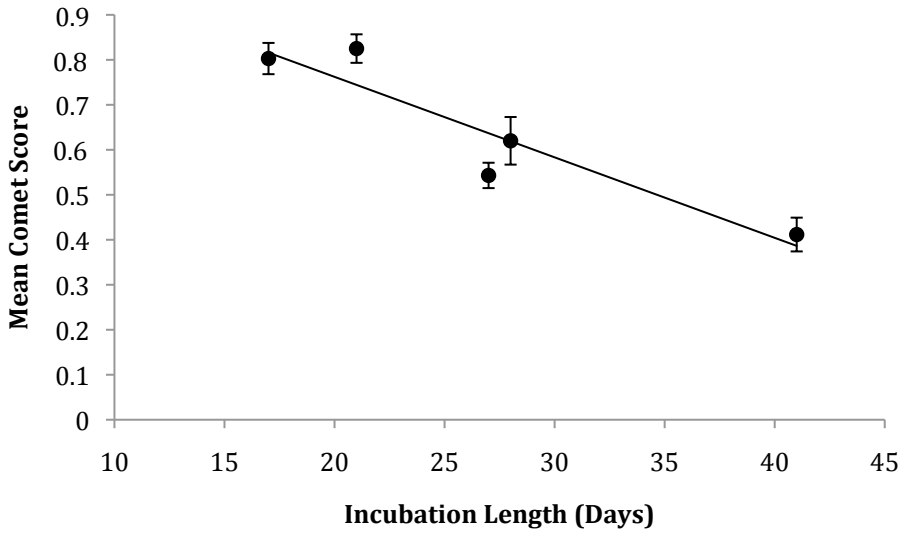


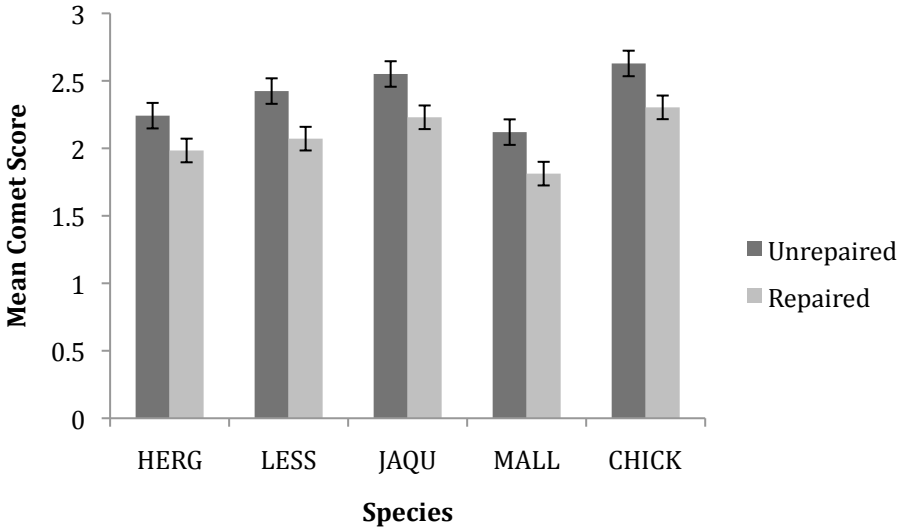
B) Heart



C)Liver


uM MDA/g lipid
0
1
2
3
4
5
6
7
8
9
10
15
20
25
30
35
40
45
Incubation Length (Days)


Mean Comet Score
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
15
20
25
30
35
40
45
Incubation Length (Days)


Mean Comet Score
3
2.5
2
1.5
1
0.5
0
HERG
LESS
JAQU
MALL
CHICK
Species
Unrepaired
Repaired