7-23-2012

Oxidative Status and Life History Trade-offs in the Cooperatively Breeding Florida Scrub-Jay

Rebecca Suzanne Heiss

OXIDATIVE STATUS AND LIFE HISTORY TRADE-OFFS IN THE COOPERATIVELY BREEDING FLORIDA SCRUB-JAY

by

Rebecca S. Heiss

A Dissertation Submitted

in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

Major: Biology

The University of Memphis

August 2012

DEDICATION

I dedicate this dissertation in honor of my grandparents Marion Heiss, Calvin Cramer, and Sandra Cramer, and in memory of Henry Heiss and Carol Cramer. Your unwavering love and support and the tenderness you have shown each of my dreams has made this possible. Thank you from the bottom of my heart.

ACKNOWLEDGEMENTS

I am overwhelmed by the number of people to whom I owe credit and thanks for the opportunities I have been given while at the University of Memphis. Above all, I must thank my dissertation advisor Dr. Stephan Schoech for his patience. Dr. Schoech went beyond expectations in his role as an advisor and has taught me more about science, writing, and life than I ever thought possible in such a short time span. I'm honored to be able to call him my mentor and friend. A huge "thank you" as well to Drs. Dave Freeman, Joel Bumgardner, Yonghong Xu, and Michael Ferkin for being excellent committee members, sounding boards, and coffee mates. Thank you to Drs. Travis Wilcoxen and Michelle Rensel (co-member of Team Awesome) who guided me in my first years in the Schoech lab and taught me how to navigate the sometimes treacherous waters of graduate school and food in the field. Michelle, your zest for life, strong determination, and the laughter we shared have brought me through more than you know. Thank you for showing me the true definition of strength. Thank you to Dr. Eli Bridge, the genius behind our *SmartFeeders* and power tool wielder extraordinaire. Many thanks to the current Schoech lab members; Emily Elderbrock, Sara Bebus, and Blake Jones for putting up with me during field seasons and always making the best of any situation. Special thanks to Dr. Tom Small for his help and humor. For struggling through it all with me, thanks especially to Sara Carter, Jared Henson, Shane Hanlon, and all of the other graduate students and faculty at the University of Memphis. Special thanks

also to the staff of the Department of Biological Sciences at the University of Memphis, especially Ms. Donna Haskins, for providing constant support.

To the staff of Archbold Biological Station, especially Dr. Hilary Swain, Sharon Hawkins, Ann Thompson, Bert Crawford, Dr. Eric Menges, Roberta Pickert, Kevin Main, Kevin Patram, Fred Lohrer, Dr. Raoul Boughton, Eric Stein, and the late Dr. Glen Woolfenden, as well as the numerous interns, thank you for providing such a rich scientific community and atmosphere. Special thanks to Dr. Reed Bowman, Lab Head of the Avian Ecology Laboratory at Archbold Biological Station, for allowing the Memphis invasion into his otherwise quiet office space. Special thanks also to Angela Tringali, Wade Ulrey, Zach Forsburg, and Rachel Hanauer, all of whom have been vital through their support, field assistance and aerobic partnership. Thank you also to Dr. Shane Pruett for his constant encouragement via target practice and having the patience of three men. Thank you to my funding sources, The American Ornithologists' Union, Sigma Xi, The University of Memphis, the National Science Foundation, and the Society for Integrative and Comparative Biology. Finally thank you to my loved ones and family, SH, SH, NW, KW, LP, DP, MH, HH, DH, CC, CC, SC, RS, CC, NC, EC, CC, NK, DC, LC, AC, MC, DS, BM , CC, JP, PS, BB, MG, ZF, and JS.

ABSTRACT

Heiss, Rebecca S., Ph.D. The University of Memphis, August, 2012. Oxidative Status and Life History Trade-offs in the Cooperatively Breeding Florida Scrub-jay. Major Professor: Stephan J. Schoech, Ph.D.

Oxidative damage has been linked to several degenerative diseases and, as such, the oxidative status of an organism has been widely used as a proxy for health state. Organisms able to resist attack by reactive oxygen species, or those with high levels of antioxidants, are typically assumed to be those in better condition (i.e., better able to withstand oxidative damage to biomolecules). Little work, however, has focused on whether the oxidative status of an organism (particularly oxidative *damage* in free-living species) influences life history decisions. My research examined the interplay between oxidative status and life history characteristics in a free-living bird, the Florida Scrub-jay (*Aphelocoma coerulescens*). I addressed the following questions: 1) Does reproductive effort covary with oxidative status? 2) Does supplementation of carotenoids (potentially important antioxidants) alleviate oxidative stress during reproduction? 3) Does supplementation of carotenoids alleviate oxidative stress during development? 4) Is oxidative status predictive of survival during early developmental stages? I found: 1) The oxidative cost of reproduction was sex specific with pre-breeding oxidative damage levels negatively correlated with reproductive effort, however, only in males. Similarly, in males, post-breeding levels of oxidative damage were significantly greater than pre-breeding levels.2) Supplementation with antioxidants did not significantly change reproductive effort, or affect post-breeding oxidative damage levels. However, there was a relationship between change in oxidative damage levels with reproductive effort

and treatment group (i. e., supplemented with antioxidants or not). Interestingly, we found no correlation between two measures of oxidative damage and a third measure of oxidative state (total antioxidant capacity). We emphasize the importance of the assessment of multiple measures of oxidative status in future studies. 3) Supplementation of nestlings did not significantly affect growth or oxidative damage measures. 4) Oxidative damage to proteins was significantly lower in older individuals, whereas TAC was significantly higher in older individuals; however, damage to DNA did not significantly differ across ages. Oxidized proteins increased significantly from the nestling to nutritional independence stages (~2 months of age) and then subsequently declined as birds reached ~9 months of age. There were no relationships between oxidized proteins and survival at these early life stages.

PREFACE

Oxidative damage to biomolecules has long been at the center of one of the most widely accepted models for aging; the free-radical theory of aging. Additionally, oxidative damage has been implicated in numerous debilitating diseases among vertebrates and in recent years has become a focus for ecologists interested in its potential to influence life histories. As an emerging field in ecology, very little work has yet been done on the impact of oxidative damage on free- living birds. The goal of my research was to determine whether oxidative damage has impacts upon reproductive decisions in adults or growth and survival of young birds in a free-living population of Florida Scrub-jays (*Aphelocoma coerulescens*). Additionally, I explored whether supplementation with antioxidants (specifically carotenoids) could alleviate the accrual of oxidative damage during both reproduction in adult birds, and growth of young. Chapter 2 ("Oxidative cost of reproduction is sex specific and correlated with reproductive effort in a cooperatively breeding bird, the Florida scrub-jay,") has been published in the journal *Physiological and Biochemical Zoology* (Heiss and Schoech, 2012). Chapter 3 ("Carotenoid supplementation, reproductive effort and oxidative damage in Florida scrub-jays,") is in review in the same journal. Chapter 4 ("Experimental supplementation of nestling Florida Scrub-jays (*Aphelocoma coerulescens*) with carotenoids has no effect on growth or oxidative state,") is formatted for submission to *Journal of Experimental Biology* to which it will be submitted in the near future. Chapter 5 ("An exploration of oxidative damage, age, and survival in the Florida scrub-jay (*Aphelocoma coerulescens*),") is in

preparation to be submitted to *The Auk*. The introduction and conclusion are formatted in the style of *Physiological and Biochemical Zoology.*

Literature Cited

Heiss, R.S., and S. J. Schoech. In Press. Oxidative cost of reproduction is sex specific and correlated with reproductive effort in a cooperatively breeding bird, the Florida scrub-jay. Physiol Biochem Zool.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

2010. Panel B shows the means and standard errors of protein carbonyl levels in nestling and independent young scrub-jays in 2011.

CHAPTER 1: INTRODUCTION

The accrual of oxidative damage and the resultant negative impacts upon an individual is at the crux of the most widely accepted theory of aging (Sohal and Weindruch 1996; Harman 1998; Finkel and Holbrook 2000; Melov et al. 2000). Differences in both the rates at which organisms accrue damage, and the mechanisms to combat such damage throughout an organisms' lifespan almost certainly contribute to among individual variations in life history decisions. In my research, I specifically addressed two major life history stages (development and reproduction) to better understand if oxidative state impacts the outcome of these major life history events.

In birds, multiple lines of evidence suggest that oxidative stress (or the resistance to it) can either predict (Bize et al. 2008), or result from reproductive effort (Alonso-Alvarez et al. 2004, 2010; Wiersma et al. 2004; Bertrand et al. 2006) and growth (Alonzo-Alvarez et al. 2007; Kim et al. 2010). However, most work on oxidative status has focused on captive individuals and has assessed limited measures of oxidative status. The goal of the dissertation research presented here was to expand this body of knowledge by assessing multiple measure of oxidative state in a free living species; as has been called for in a recent review (Metcalfe and Alonso-Alvarez 2010).

The population of Florida scrub-jays (*Aphelocoma coerulescens*) used in this study resides at Archbold Biological Station and has been intensely studied for over 20 years (e.g., Schoech et al. 1991). Florida scrub-jays provide a unique opportunity to study oxidative damage in a long-lived organism in their natural habitat. Their sedentary nature and our continuous monitoring of this marked

population has allowed us to track individuals throughout their lifetimes to assess both immediate and longer-term impacts of oxidative damage

In the first two studies presented (Chapters 2 and 3), I sought to uncover whether oxidative damage interacted with reproductive effort in adult Florida scrub-jays. Specifically, I examined the impacts of oxidative damage on reproductive effort (and reproductive effort on oxidative damage), including an antioxidant supplementation experiment designed to reduce oxidative stress in selected birds during the breeding season. I then proceeded to explore the relationship between oxidative status and growth during early developmental stages (Chapter 4). Finally, I assessed if age and oxidative status were related and whether oxidative status during early development was predictive of survival (Chapter 5).

References

Alonso-Alvarez C., S. Bertrand, G. Devevey, J. Prost, B. Faivre, O. Chastel, and G. Sorci. 2006. An experimental manipulation of life-history trajectories and resistance to oxidative stress. Evolution 60:1913-1924.

Alonso-Alvarez C., S. Bertrand, G. Devevey, J. Prost, B. Faivre, and G. Sorci. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecol Lett 7:363-368.

Bertrand S., C. Alonso-Alvarez, G. Devevey, B. Faivre, J. Prost, and G. Sorci. 2006. Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. Oecologia 147:576-584.

Bize P., G. Devevey, P. Monaghan, B. Doligez, and P. Christe. 2008 Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. Ecology 89: 2584-2593.

Finkel T., and N.J. Holbrook. 2000. Oxidants, oxidative stress and the biology of ageing. Nature. 408: 239–247.

Harman D. 1998. Aging and oxidative stress. J Int Fed Clin Chem 10:24–27.

Kim S-Y., J. C. Noguera, J. Morales, and A. Velando. 2010. Quantitative genetic evidence for trade-off between growth and resistance to oxidative stress in a wild bird. Evol Ecol 25: 461–472.

Melov S, J. Ravenscroft , S. Malik, M. S. Gill, D. W. Walker, P.E. Clayton, D. C. Wallace, B. Malfroy, S. R. Doctrow, and G. J. Lithgow. 2000. Extension of life-span with superoxide dismutase/catalase mimetics. Science 289: 1567–1569.

Metcalfe N. B., and C. Alonso-Alvarez. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct Ecol 24:984-996.

Schoech S. J., R. L. Mumme, and M. C. Moore. 1991. Reproductive endocrinology and mechanisms of breeding inhibition in cooperatively breeding Florida Scrub Jays (*Aphelocoma c. coerulescens*). Condor 93:354–364.

Sohal R. S., and R. Weindruch. 1996. Oxidative stress, caloric restriction, and aging. Science 273:59–63.

Wiersma P., C. Selman, J. R. Speakman, and S. Verhulst. 2004. Birds sacrifice oxidative protection for reproduction. Biol Lett 271:S360-S363.

CHAPTER 2: OXIDATIVE COST OF REPRODUCTION IS SEX-SPECIFIC AND CORRELATED WITH REPRODUCTIVE EFFORT IN A COOPERATIVELY BREEDING BIRD, THE FLORIDA SCRUB-JAY

Introduction

A number of factors have been assessed to further our understanding of how organisms balance self-maintenance and investment in offspring. Increasingly, oxidative balance has been viewed as an important regulator of life history decisions (Monaghan et al. 2009). Reactive Oxygen Species (ROS), which are continuously formed as byproducts of cellular metabolism, must be balanced by antioxidant defenses (e.g., dietary antioxidants and endogenously produced antioxidant enzymes) that prevent damage to biomolecules (Davies 2000; Halliwell and Gutteridge 2007). Increased energetic demands and the associated increase in metabolism during a breeding season (Verhulst and Tinbergen 1997; Nilsson 2002; although see Williams and Vézina 2001), have the potential to shift the oxidative balance of an organism in favor of ROS and, thereby result in substantial oxidative damage (Halliwell and Gutteridge 2007). The hypothesis that individuals face a trade-off between investment in reproduction and oxidative self-maintenance has been tested in captive birds (Wiersma et al. 2004; Alonso-Alvarez et al. 2004, 2010); however, to the best of our knowledge only two other studies have explored this relationship in free-living birds (Losdat et al. 2011; van de Crommenacker et al. in press).

Whereas the aforementioned studies of Losdat et al. (2011) and van de Crommenacker et al. (in press) elegantly showed that increased reproductive effort resulted in reduced antioxidant capacity and increased oxidative stress,

respectively, we focused on the resulting *damage* to a specific class of biomolecule (protein). Such damage could be the result of either a reduction in antioxidant capacity, an increased production of ROS, or a combination of the preceding. Further, by measuring oxidative damage levels pre- and post-reproduction, we were able to assess both whether oxidative state: 1) predicted or 2) varied as a result of subsequent reproductive effort.

Materials and Methods

Study Species: the Florida scrub-jay

The study population of Florida scrub-jays is located at Archbold Biological Station, Highlands County, Florida (27°10'N, 81°21'W, elevation 38–68 m). All individuals are uniquely marked with colored plastic leg bands, and an aluminum numbered U.S. Geological Survey band that allow us to track individuals throughout their lifetime. Only female breeders incubate eggs and brood nestlings. True double-brooding occurs infrequently (less than 4% of the time), and no data from double broods were included in these analyses (Woolfenden and Fitzpatrick 1996; Schoech 2010).

Trapping and Blood Sample Collection

In 2010, adult birds (both helpers and breeders, although only breeders were of interest to this study) were trapped in monitored Potter traps from late January to late February to obtain, pre-breeding plasma samples and from mid-April through mid-June, after fledging young for post-breeding samples. Each morning between 700-1100h, traps were baited with peanuts and watched continuously until capture of an individual. Traps were removed after capture. Following

brachial venipuncture with a 25-gauge needle, blood samples were collected (~150 µL) in microhematocrit tubes within 2 min of capture. Samples were placed on ice and, upon return to the lab (within 1 – 3 h), were centrifuged to remove the plasma portion, that was then frozen and stored at -20^{o} C until analysis for levels of oxidative damage to proteins. All trapping and bleeding procedures were done under federal and state permits to SJS (TE117769-3 and WX08091A, respectively) and followed University of Memphis IACUC approval to RSH (#0667).

Protein Carbonyl Assay

Direct or indirect formation of protein carbonyl groups result from bond cleavage by ROS or the attachment of lipid peroxidation byproducts, respectively (Mateos and Bravo 2007). Because protein carbonyls form relatively early in an individual experiencing oxidative stress and are stable, they are a widely used, tractable biomarker of oxidative damage (Monaghan et al. 2009).

Additionally, oxidative damage to proteins may be of particular relevance to biological systems given that the only true enzymatic repair system known for oxidized proteins is specific to the restoration of Met from Met sulfoxide, and Cys sulfhydryls from disulfides (Shacter 2000). While oxidized proteins can be removed from tissues via proteolysis, they can also evade detection and go unrecognized (Sahakian et al. 1995), and this may lead to the accumulation of oxidized proteins, which is often associated with disease and ageing (Oliver et al. 1987; Davies 1990).

Nine protein carbonyl assay kits (Cayman Chemical Company #10005020) were used to assay all samples (intra-assay CVs based on standards: 1.3–4.3%). Briefly, a reaction between protein carbonyls and 2,4-dinitrophenylhydrazine (DNPH) produces a protein–hydrazone that is quantified spectrophotometrically (at 360nm) and standardized to the samples' specific protein concentration. Sample protein concentration was quantified spectrophotometrically at 280nm from a bovine serum albumin standard curve.

Statistical Analysis

We estimated paternal effort by summing the total number of days that a breeder cared for offspring prior to fledging, adjusted for the number of offspring that were present (e.g. 4 offspring for 18 days = 4*18 = 72). Reproductive effort also included a sum of the days for which males provided food to the incubating female, or for females, a sum of the days during which she incubated (given that this was only a single additional mouth to feed, this number was not corrected for the number of eggs present; Wilcoxen et al. 2010). Nests are monitored regularly throughout the breeding season. We check completed nests every other day until the first egg is laid, and every 3-4 days during incubation to assure continuance and collect incubation day data. After hatching, nests are checked at least every three days to confirm that the nest is still active and, if so, to determine the number of nestlings present. Given that Florida scrub-jays are cooperative breeders, the presence of helpers might also require consideration when quantifying reproductive effort of male breeders. However, Mumme (1992) found that the presence of helpers in the Florida scrub-jay system did not

significantly change the feeding rates of the breeding pair. As such, we felt confident in our exclusion of helpers from our measure of the reproductive effort of breeder males. While we are confident that our index of “offspring days” is an appropriate measure of reproductive effort, we also assessed other measures of reproduction including clutch size and number of nestlings produced. Analyses that used these alternative measures yielded qualitatively similar findings to those produced using the above described index of reproductive effort.

A Repeated Measures General Linear Model was used to assess protein carbonyl damage prior to, and post-reproduction, controlling for sex and reproductive effort. The model initially included age and days between samples (i.e., elapsed days between the pre- and post-breeding samples), however, these non-significant variables were removed using a backward removal method based on non-significant P-values. A General Linear Model was used to assess whether pre-breeding oxidative damage levels were related to subsequent reproductive effort with sex as a fixed factor. As above, age and days between samples were included but subsequently removed based on non-significant P-values.

Results

The examination of pre- and post-breeding oxidative damage levels revealed a significant sex*time (from pre- to post-breeding) interaction (see Table 1)

.

Table 1: Final model describing differences in pre- and post- breeding oxidative damage levels to protein (Time) in breeder Florida scrub-jays (df = 25). From this model, the significant interaction between Time and Sex was explored through post-hoc analysis.

Variable	**F**	**P-value**
Time	175.29	< 0.001
Sex	8.47	0.007
Time* Sex	8.47	0.007

Post-hoc exploration by sex revealed that post-breeding oxidative damage levels of males were markedly greater than pre-breeding levels ($F_{1,18}$ = 6.65, P = 0.02), whereas females exhibited no change ($F_{1,10}$= 0.12, P = 0.73; see Fig 1).

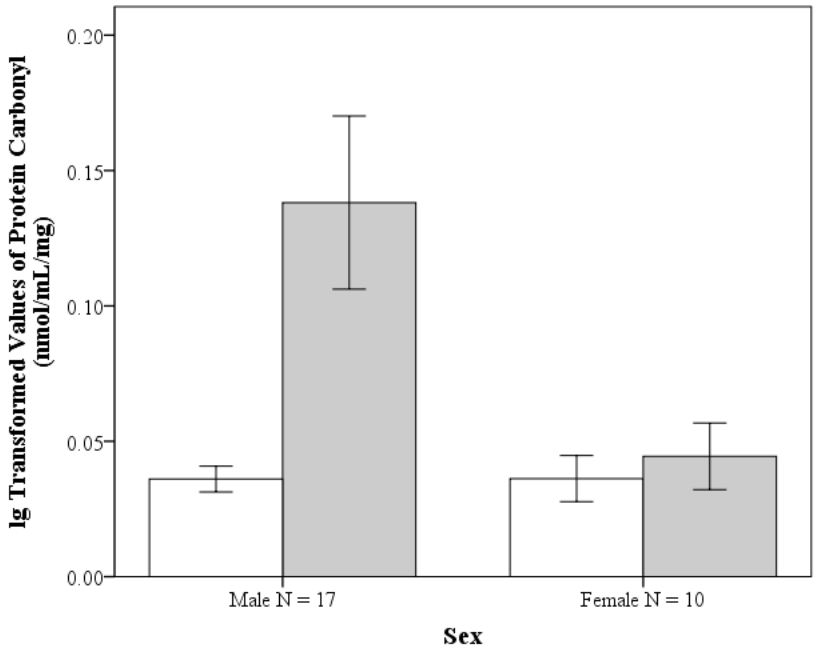


Figure 1: Protein oxidative damage levels pre- and post- breeding by sex of breeder Florida scrub-jays. White bars show pre-breeding, black bars represent post-breeding. Error bars show $\overline{X}$± SE.

Examination of whether pre-breeding levels of oxidative damage predicted reproductive effort noted a significant interaction between sex and reproductive effort (see Table 2).

Table 2: Final model describing relationship between pre-breeding oxidative damage levels to proteins and reproductive effort in breeder Florida scrub-jays (df = 31). The significant interaction between sex and pre-breeding oxidative damage to proteins was explored through post-hoc analysis.

Variable	F	P-value
Sex	10.22	< 0.01
Pre-breeding Oxidative Damage	4.21	0.05
Sex* Pre-breeding Oxidative Damage	9.24	0.01

Post-hoc tests (GLMs restricted by sex) revealed a significant relationship between pre-breeding levels of oxidative damage and reproductive effort in males ($F_{1,18} = 5.52$, $P = 0.03$, $r^2 = 0.25$), but not in females ($F_{1,16} = 1.09$, $P = 0.31$, $r^2 = 0.07$; see Fig 2).

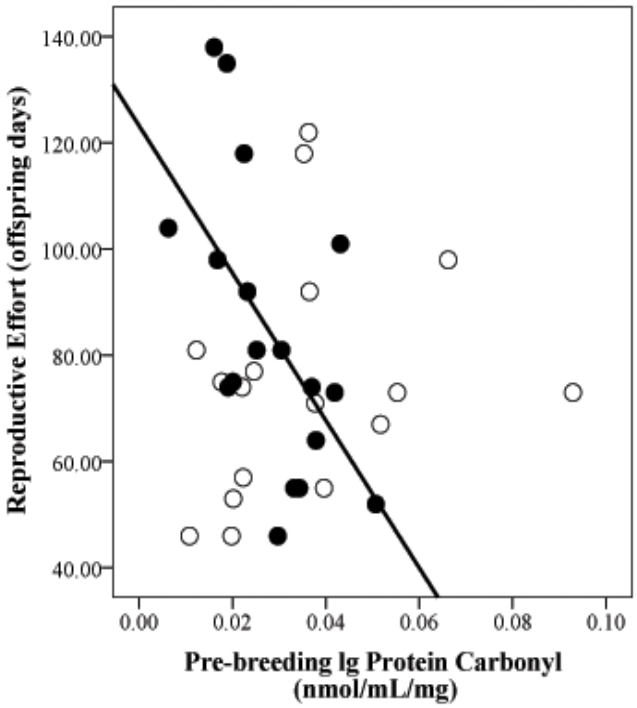


Figure 2: Pre-breeding protein oxidative damage levels of male Florida scrub-jay breeders (black circles, solid line) were significantly related to reproductive effort ("offspring days," number of days offspring were cared for multiplied by the number of offspring present), whereas there was no relationship for female breeders.

Discussion

The hypothesis that oxidative stress is a major cost of reproduction is not new (Salmon et al. 2001); however, little research has investigated this in free-living populations (Metcalfe and Alonso-Alvarez 2010). Here we show that not only does oxidative damage apparently result from reproduction in a sex dependent manner, but that an accumulation of oxidative damage may predict subsequent male reproductive effort.

Captive studies have demonstrated that experimentally increased reproductive effort, via clutch- or brood-size manipulation, resulted in reduced oxidative protection in birds (Alonso-Alvarez et al. 2004; Wiersma et al. 2004). Our finding that oxidative damage was significantly increased in males after the breeding period is consistent with previous work in which free-living male, but not female, Eurasian kestrels (*Falco tinnunculus tinnunculus*) exhibited increased levels of oxidative stress during the nestling rearing stage (Casagrande et al. 2011). Similar to the patterns of paternal behavior observed in the Eurasian kestrel, male Florida scrub-jays provide food to the female during incubation and provision both the female and young during the first week or more of the nestling stage when females extensively brood nestlings. For example, video monitoring of nests when nestlings were from 3 – 5 days of age in 2010 found a significant difference in the mean number of feeding visits made by males (6.7/hr) compared with females (2.3/hr, Small and Schoech, unpublished data). We suggest that in species in which males provision their mates and are largely responsible for provisioning young, such as the Florida scrub-jay, such sex specific patterns of reproductive associated oxidative damage will be the norm. It would be of interest to conduct studies across species from across the spectrum of parental care ranging from solely paternal care to exclusively maternal care. Further, altering the reproductive effort required by males or females at the extremes of this spectrum through manipulations of clutch size or resource availability would allow us to more conclusively determine whether sex-specific

oxidative damage resulted from differences in reproductive effort as opposed to other factors.

Because they produce far larger gametes than males, females have long been considered to be the sex that invests the most in reproduction; however, most estimates of the energetic costs for egg production in passerines are relatively low (13-41% of basal metabolic rate [BMR]) (Monaghan and Nager 1997) when compared to the costs of nestling rearing (e. g., 380% of BMR in barn swallows, *Hirundo rustica,* (Ward 1996). Given these data, it may be that the energy expended by a female Florida scrub-jay is less than that of her mate. Alternatively, there may be a sex-based difference in either the capability to combat ROS or the ability to repair damaged biomolecules.

Given that the increased provisioning effort by males is in addition to what is required for self-maintenance, the combined energetic expenditure may be responsible for the observed increased oxidative damage levels incurred during the breeding season. In general, increased energetic demands and metabolism during reproduction result in increased ROS production, which may in turn lead to an oxidative imbalance (Alonso-Alvarez et al. 2004, 2010; Wiersma et al. 2004). Specifically, the greater amount of flight time needed to meet increased provisioning demands may be responsible for this imbalance, as flight has been shown to increase oxidative damage in adult budgerigars (*Melopsittacus undulatus*) (Larcombe et al. 2008).

Our finding that oxidative damage levels of males prior to breeding are ‘predictive’ of subsequent reproductive effort supports the hypothesis that

oxidative stress plays a role in trade-offs that shape life histories (Costantini 2008; Monaghan et al. 2009). Individual male scrub-jays in better oxidative condition entering the breeding season invested more in reproductive effort. Numerous factors, including but not limited to age and reproductive history (Alonso-Alvarez et al. 2010), food availability (Giroud et al. 2009), and stressful environmental conditions (Berglund et al. 2007; Costantini et al. 2011) all can affect the accumulation of oxidative damage in an individual. Further studies in which some of these factors are manipulated prior to and during the breeding season would greatly enhance our understanding of the role of oxidative stress in mediating reproductive decisions.

Acknowledgements

We thank all at Archbold Biological Station, especially R. Bowman and H. Swain. Special thanks to T. W. Small, M. A. Rensel, and R. Hanauer. We further thank the anonymous reviewers whose contributions greatly improved the quality of this manuscript. This research was funded by a National Science Foundation collaborative grant to S.J. Schoech and E. S. Bridge (IOS-0919899 and -0919528).

Literature Cited

Alonso-Alvarez C., S. Bertrand, G. Devevey, J. Prost, B. Faivre, and G. Sorci. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecol Lett 7:363-368.

Alonso-Alvarez, C., L. Pérez-Rodríguez, J. T. García, J. Viñuela, and R. Mateo. 2010. Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. Physiol Biochem Zool 83:110-118.

Berglund, A. M. M., J. Sturve, L. Förlin, and N. E. I. Nyholm. 2007. Oxidative stress in pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environ Res 105:330-339.

Casagrande, S., G. Dell'omo, D. Costantini, J. Tagliavini, and T. Groothuis. 2011. Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel: a multi-factorial study. Comp Biochem Phys A 160:16-26.

Costantini, D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett 11:1238-1251.
Costantini, D., V. Marasco, and A. P. Møller. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J Comp Physio B 181:447-456.

van de Crommenacker, J., D. S. Richardson, A. M. Koltz, K. Hutchings, and J. Komdeur. In press. Parasitic infection and oxidative status are associated and vary with breeding activity in the Seychelles warbler. Proc R Soc Lond B.

Davies, K. J. A. 1990. Protein oxidation and proteolytic degradation. General aspects and relationship to cataract formation. Adv Exp Med Biol 264:503–511.

Davies, K. J. A. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair and replacement systems. IUBMB Life 50:279-289.

Giroud, S., M. Perret, C. Gilbert, A. Zahariev, J. Goudable, Y. Le Maho, H. Oudart, I. Momken, F. Aujard, and S. Blanc. 2009. Dietary palmitate and linoleate oxidations, oxidative stress, and DNA damage differ according to season in mouse lemurs exposed to a chronic food deprivation. Am J Physiol-Reg I 297:R950-R959.

Halliwell, B. and J. Gutteridge. 2007. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, UK.

Larcombe, S. D., C. A. Tregaskes, J. S. Coffey, A. E. Stevenson, L. Alexander, and K. E. Arnold. 2008. The effects of short-term antioxidant supplementation on

oxidative stress and flight performance in adult budgerigars *Melopsittacus undulatus*. J Exp Biol 211:2859-2864.

Losdat, S., F. Helfenstein, B. Gaude, and H. Richner. 2011. Reproductive effort transiently reduces antioxidant capacity in a wild bird. Behav Ecol 22:1218-1226.

Mateos, R. and L. Bravo. 2007. Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). J Sep Sci 30:175-191.

Metcalfe, N. B. and C. Alonso-Alvarez. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct Ecol 24:984-996.

Monaghan, P. and R. G. Nager. 1997. Why don't birds lay more eggs? TREE 12:270-274.

Monaghan, P., N. B. Metcalfe, and R. Torres. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett 12:75-92.

Mumme, R. L. 1992. Do helpers increase reproductive success? An experimental analysis in the Florida scrub jay. Behav Ecol Sociobiol 31:319–328.

Nilsson, J. A. 2002. Metabolic consequences of hard work. Proc R Soc Lond B 269:1735-1739.

Oliver, C. N., B. W. Ahn, E. J. Moerman, S. Goldstein, E. R. Stadtman. 1987. Age-related changes in oxidized proteins. J Biol Chem 262:5488–5491.

Sahakian, J. A., L. I. Szweda, B. Friguet, K. Kitani, and R. L. Levine. 1995. Aging of the liver: Proteolysis of oxidatively modified glutamine synthetase. Arch Biochem Biophys 318: 411–417.

Salmon, A. B., D. B. Marx, and L. G. Harshman. 2001. A cost of reproduction in *Drosophila melanogaster*: stress susceptibility. Evolution 55:1600-1608.

Schoech, S. J. 2010. A reply to Dhondt: Broodedness and latitude affect the response of reproductive timing of birds to food supplementation. J Ornithol 151:959-961.

Shacter, E. Quantification and significance of protein oxidation in biological samples. 2000. Drug Metab Rev 32:307–326.

Verhulst, S. and J. Tinbergen. 1997. Clutch size and parental effort in the great tit *Parsus major*. Ardea 85:111-126.

Ward, S. 1996. Energy expenditure of female barn swallows *Hirundo rustica* during egg formation. Physiol Zool 69:930-951.

Wiersma, P., C. Selman, J. R. Speakman, and S. Verhulst. 2004. Birds sacrifice oxidative protection for reproduction. Biol Lett 271:S360-S363.

Wilcoxen, T. E., R. K. Boughton, and S. J. Schoech. 2010. Selection on innate immunity and body condition in Florida scrub-jays throughout an epidemic. Biol Lett 6:552-554.

Williams, T. D. and Vézina, F. 2001. Reproductive energy expenditure, intraspecific variation, and fitness. Curr Ornithol 16: 355–405.

Woolfenden, G. E. and J. W. Fitzpatrick. 1996. Florida Scrub-jay (*Aphelocoma coerulescens*), The Birds of North America Online (A. Poole and F. Gill, Eds.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online.

CHAPTER 3: CAROTENOID SUPPLEMENTATION, REPRODUCTIVE EFFORT, AND OXIDATIVE DAMAGE IN FLORIDA SCRUB-JAYS

INTRODUCTION

The oxidative balance of an organism has been proposed to be a major determinant of decisions (Monahan et al., 2009). During times of increased energetic demand, such as reproduction, increased rates of metabolism result in greater production of reactive oxygen species (ROS) that can cause damage to biomolecules if not countered by antioxidant defenses (Halliwell and Gutteridge, '99; Davies, 2000; Nilsson, 2002). The potential damage caused by ROS can be extensive, as oxidative damage has been implicated in senescence, as well as numerous diseases among vertebrates (Beckman and Ames, '98; Finkel and Holbrook, 2000; Golden et al., 2002; Dalle-Donne et al., 2006). Therefore, one would expect an organism to adjust its investments in self-maintenance and reproduction and, thereby maximize fitness.

Florida scrub-jays (*Aphelocoma coerulescens*) are relatively long-lived (with a maximum lifespan of 15.5 years), cooperatively breeding birds, individuals of which typically spend their entire lives within a kilometer of their natal territory (Woolfenden and Fitzpatick, '96). While females and non-breeding helpers (typically yearling offspring, that are present in approximately half of the territories) help to feed nestlings beginning when the young are approximately a week old, the provisioning until then, including provisioning of the incubating and brooding female, is done by the male breeder (Schoech et al., '96; Woolfenden and Fitzpatick, '96; Wilcoxen et al., 2010). Oxidative damage to proteins in male,

but not female, breeders was significantly increased after reproduction in Florida scrub-jays (Heiss and Schoech, *in review*). For birds rearing nestlings, increases in basal metabolic rate (BMR) range from 3.2 - 4.0 x BMR (Drent and Daan, '80; Peterson et al., '90; Nilsson and Råberg, 2001). Given the high energetic cost to raise offspring, it is of little surprise to find that during this time, males trade-off oxidative balance in favor of investment in reproduction.

Although a number of studies have demonstrated that breeding effort results in increased oxidative stress in birds (e. g., Alonso-Alvarez et al., 2004, 2006, 2010; Wiersma et al., 2004), the role of antioxidants in prevention of damage is unclear. Specifically, carotenoids, non-synthesizable pigments with antioxidant properties, have gained attention for their role in oxidative balance. Despite a recent review, in which Costantini and Møller (2008) state that carotenoids are of minor importance as antioxidants in birds, Betrand et al. (2006), demonstrated that supplemental carotenoids were able to reverse the negative correlation of reproductive effort and resistance to oxidative stress in zebra finches (*Taeniopygia guttata*). It is important to point out that, nearly all of the research on carotenoids as antioxidants have focused on species that depend upon carotenoids for plumage coloration, thereby confounding interpretation of the results (Mougeot et al., 2009; Casagrande et al., 2011; Losdot et al., 2011). To the best of our knowledge, ours is the first research to address the effects of carotenoids on oxidative balance during reproduction in a species in which plumage coloration is based largely on structure, rather than carotenoid-based pigments. We suspect that carotenoids may function distinctly

differently in birds that are not reliant on them for plumage coloration, given that the tradeoff between coloration (used for example in honest signaling) and oxidative balance is irrelevant in this study species (see Alonzo-Alvarez et al., 2008).

There are scores of methods for assessing various aspects of oxidative balance (for reviews see Mateos and Bravo, 2007; Somogyi et al., 2007), however, most physiological ecologists have focused on measures of ROS, and some measure of antioxidants, to estimate levels of oxidative stress experienced by an individual. Most studies that have assessed whether reproduction affects oxidative balance have failed to specifically measure oxidative *damage* (for review see Metcalf and Alonso-Alvarez, 2010). While several recent studies have measured derivatives of reactive oxygen metabolites (dROMS), which are indicative of free radical formation and the magnitude of these radicals within a biological sample (Costantini et al. 2010; Casagrande et al. 2011; van de Crommenaker et al. 2011), to our knowledge, our previous work on Florida scrub-jays is the only other study to have measured actual markers of oxidative damage in association with reproduction in a free-living bird (Heiss and Schoech, *in review*).

For this study we assess multiple measures of oxidative state, including damage to proteins and DNA, as well as non-enzymatic Total Antioxidant Capacity (TAC) of plasma. In this way, we consider the understudied links between oxidative damage and reproduction in free-living birds. Further, our assessment of TAC as a third measure of oxidative state allows for comparison

among other studies that have used, or will use, this as a proxy for estimating the resistance of an individual to oxidative damage.

Specifically, our goals were twofold. First, to determine whether supplemental antioxidants provided to male Florida scrub-jays could negate the resulting reproduction-related, oxidative damage. Second, as stated above, to determine whether measures of oxidative damage and TAC are correlated in a free-living bird.

MATERIALS AND METHODS

Study species and site

Research was conducted at Archbold Biological Station (ABS), Highlands County, Florida, USA 27°10'N, 81°21'W, elevation 38–68 m; for further detail see Schoech ('96) and Schoech and Bowman (2003). Because Florida scrub-jays are a non-migratory, highly sedentary species, virtually all individuals in our study spend their entire lives at ABS. Each scrub-jay in this population is uniquely marked with coloured plastic leg rings, and a numbered aluminum U.S. Geological Survey ring which allow us to track individuals throughout their lifetimes. In addition, an NSF-funded collaborative grant to Schoech and Bridge (IOS-0919899 and -0919528) has allowed our study population to be tagged with Passive Integrated Transponders (PIT tags) that permit specific birds to be supplemented by Radio Frequency Identification-enabled (RFID) *SmartFeeders*. All male breeders for this study were fitted with PIT tags.

Experimental design and antioxidant manipulation

Male breeders were randomly assigned to one of three treatment groups: 1) males supplemented with high protein/high fat food (N=10); 2) males supplemented with high protein/high fat food enhanced with antioxidants (N=8); or 3) control males, not supplemented (N=10). Sample size in the antioxidant group was unfortunately lower due to differences in the willingness of certain birds to approach and use feeders. Lutein and zeaxanthin were chosen as the supplemental carotenoids because these antioxidants have been commonly used in experiments to reduce oxidative stress (Bertrand et al., 2006; Costantini et al., 2007; Larcombe et al., 2008; Helfenstein et al., 2010). Additionally, Heiss et al. (2011) found these to be the most abundant carotenoids in Florida scrub-jay plasma. Eukanuba© Chicken Formula Kitten Food (that also served as treatment #1 above) was coated in a mixture of lutein and zeaxanthin (Kemin Foods, L.C., FloraGLO Lutein, Des Moines, Iowa, USA), to approximate an appropriate dose of each carotenoid based upon calculations in Biard et al. (2006) and *SmartFeeder* usage by the jays in 2010. Each food pellet was coated with approximately 3.5 µg of lutein and 0.02 µg zeaxanthin. Confirmation of estimated antioxidant content per pellet was tested in an outside lab (D. Kopsell, University of Tennessee) to confirm both the estimated dosage and stability of the carotenoids over the course of the maximal two week period from application of the carotenoid until consumption by the jays.

Feeders were deployed at each territory between the 14-16th of January 2011. Regular (non-antioxidant enhanced) kitten food was provided in each of the feeders and all birds were allowed to feed *ad libitum* for an average of one

week to allow birds to become familiar with, and learn to use, the feeders. Territories were assigned to a treatment group (kitten food or antioxidant enhanced kitten food) from 1-18th of February, and the *SmartFeeders* were programmed to allow only access by breeder males for the remainder of the breeding season. All birds received supplemental diets until hatching of their clutch was completed, at which point all feeders were switched to dispense untreated mealworms, an appropriate food for nestlings. Mealworms were dispensed from feeders to selected males until the brood had fledged. In the case of nest failures, feeders were switched back to dispense kitten food (either regular or antioxidant enhanced) until hatching of the replacement clutch (both the incubation and nestling periods are approximately 18 days; Woolfenden and Fitpatrick, 1984) . Any visit to a *SmartFeeder* by a RFID-permitted bird in either of the two supplemented groups resulted in the dispensing of a single pellet of kitten food. The identity and mass of birds are electronically recorded at each visit. There were no differences in the average number of visits made to the feeders between the two supplemented groups ($t_{1,16}$ = 1.35, P = 0.20).

Trapping and blood sample collection

Adult birds were trapped in continually monitored Potter or drop traps from late January through mid-February 2011 (before territories were assigned to treatment group) to obtain, pre-breeding blood samples. Jays were recaptured using the same methods, from mid-April through mid-June 2011, after they had fledged young. Following brachial venipuncture with a 25-gauge needle, blood samples were collected (~150 µL) in heparinized microhematocrit tubes within

120 seconds of capture, thus minimizing the likelihood that blood chemistry values would be altered due to the stress of capture. Blood samples were kept chilled with 'cool-packs' (also known as 'Blue-Ice^{TM}') in an insulated bag until return to the laboratory (within1-3 h). Samples were immediately centrifuged allowing separation of the cellular and plasma portions. Plasma was drawn off and stored at -80^{o} C until analysis for TAC and measures of oxidative damage.

Morphometric data (body mass, head breadth, distance from the nares to bill tip, and lengths of the wing-cord, head-plus-bill, culmen, and tail) were taken following blood sampling (see Schoech et al., '96 for further details). To control for diel fluctuations in physiologic measures, all blood samples were collected between 0700 and 1100.

Measures of oxidative state

Because protein carbonyls form relatively early in an individual experiencing oxidative stress and are stable, they are a widely used, tractable biomarker of oxidative damage (Monaghan et al., 2009). Nine protein carbonyl assay kits (Cayman Chemical Company #10005020) were used to assay all samples run in duplicate (intra-assay CVs based on standards: 1.3-4.3%, Cayman notes that inter-assay CV is 8.5%). Briefly, 40µL of plasma were diluted into 410µL of a phosphate buffered saline solution. Diluted plasma and controls were reacted with a 2,4-dinitrophenylhydrazine (DNPH) to produce a protein-hydrazone that was then quantified spectrophotometrically (at 360nm) and standardized to the samples' specific protein concentration. Sample protein concentration was

quantified spectrophotometrically at 280nm from a bovine serum albumin standard curve.

Oxidative damage to DNA was estimated via the concentration of the oxidized derivative of guanine, 8-hydroxy-2'-deoxyguanosine (8-OHdG). Briefly, 50µL of plasma (or standard) were added to an 8-OHdG conjugate-coated plate, and incubated at room temperature for 10 minutes. An anti-8-OHdG monoclonal antibody was added, followed by addition of diluted secondary antibody-enzyme conjugate. Following an hour incubation and triple wash, the absorbance of each micro-well was determined at 450 nm (Cell BioLabs, Inc; OxiSelect™ Oxidative DNA Damage ELISA Kit [8-OHdG Quantitation] STA-320: [Shen et al., 2007]). A total of three assay kits were used (intra-assay CVs based on standards: 3.5-6.9%, inter-assay CV = 9.0%).

Finally, the Oxy-Adsorbent test (Diacron International, Grosseto, Italy), provided a quantitative measure of total antioxidant capacity by evaluating the ability of plasma to withstand a controlled free radical attack by hypochlorous acid (HClO; a relevant biological oxidant (Costantini and Dell'Omo, 2006a,b; Costantini et al., 2006). Briefly, 10µL of plasma were diluted 1:100 with distilled water. The diluted plasma was incubated for 10 minutes at 37^{o}C with 200µL of a HClO solution before 5µL of N, N-diethyl-p-phenylenediamine (a chromogen) were added. Immediately thereafter, absorbance was read at 505 nm and compared with standards. The antioxidant capacity is expressed as micromoles of neutralized HClO per mL of sample. It is important to note that while other methods of measuring plasma antioxidant capacity have been criticized for their

overemphasis of the contributions of uric acid, the contribution of uric acid to TAC as measured by this assay is low (Costantini, 2010). A total of six assay kits were used (intra-assay CVs based on standards: 7.1-10.6%, inter-assay CV = 13.0%).

Statistical analyses

Initial variability among birds in different treatment groups at the beginning of the study was assessed in multiple ANOVAs to compare all measures of oxidative state and initial pre-breeding mass.

To assess correlations among measures of oxidative state, we ran two, two-tailed Pearson's correlations. The first correlation was analysed with pre-breeding measures of damage to DNA, damage to proteins, and TAC, whereas the second assessed post-breeding measures of these same parameters. To assure treatment group did not influence these correlations, we used the standardized residuals from ANOVA models that controlled for treatment in a second set of two-tailed Pearson's correlations. Because treatment did not have any effect on these measures, only the initial two-tailed Pearson's correlations are presented below.

Reproductive effort was estimated by summing the total days that a breeder cared for offspring prior to fledging, corrected for the number of offspring present on each day (see Wilcoxen et al., 2010; Heiss and Schoech, *in review*). As a second measure of reproductive effort we also measured male mass prior to and post-breeding. We used ANOVA to compare reproductive effort among groups.

We used backwards removal General Linear Models (GLMs) to assess differences between post-breeding oxidative damage levels and post-breeding TAC among treatment groups. In each model we entered pre-breeding levels of the respective oxidative state measure, change in mass, and reproductive effort as covariates, with treatment as a fixed factor. A factor analysis was used to combine the changes in oxidative damage to proteins and DNA from pre- to post-breeding. We used the first component from this analysis (Eigenvalue = 1.03, explained 51.6% of the variance) in a GLM to determine any differences in overall pre- to post-breeding oxidative damage levels among treatment groups. Reproductive effort and change in mass were included as covariates. We used repeated measures GLMs to determine whether the three measures of oxidative state differed from pre- to post-breeding. In each analysis, mass change and reproductive effort were included as covariates, with treatment as a fixed factor. Day-of-year was initially included in all of the above models but later removed as a non-significant factor in each model based upon its non-significant *P*-value (i.e., $P > 0.05$).

RESULTS

No initial differences existed between treatment groups in pre-breeding mass ($F_{2,13} = 0.67$, $P = 0.53$), oxidative damage to proteins ($F_{2,13} = 0.33$, $P = 0.72$) or DNA ($F_{2,12} = 0.21$, $P = 0.82$), or in TAC ($F_{2,12} = 0.52$, $P = 0.61$).

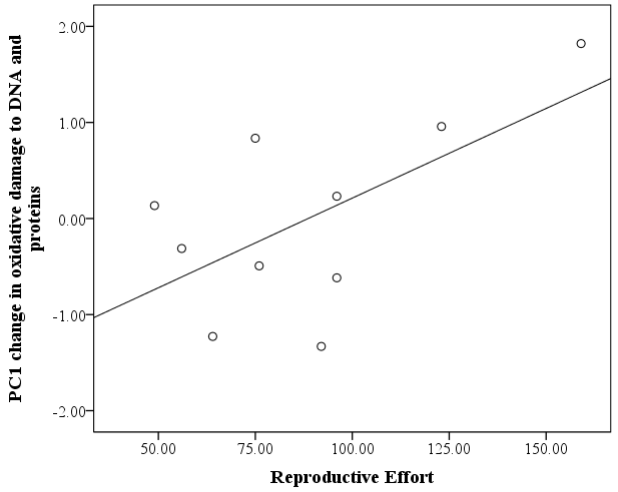
There were no correlations between any measures of oxidative state in either pre- or post-breeding measures (Table 1).

Table 1. Measures of oxidative stress in male Florida scrub-jays were not correlated with one another at either pre-breeding or post-breeding.

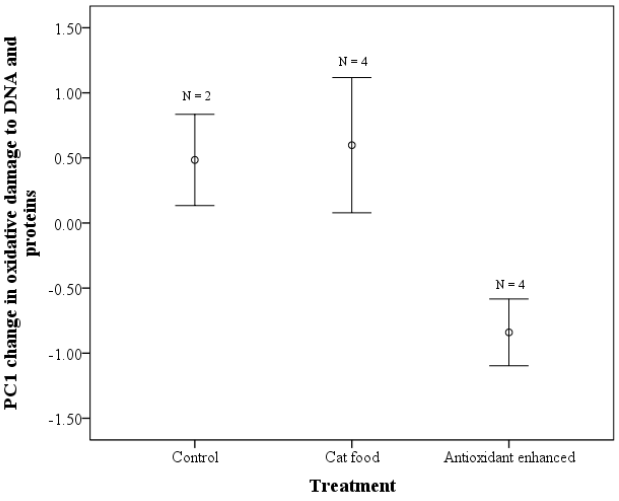
	Pre-Breeding			Post-Breeding		
	Pearson's r	**P-value**	**N**	**Pearson's r**	**P-value**	**N**
Protein/TAC	0.15	0.66	11	0.18	0.50	16
DNA/Protein	-0.04	0.91	11	0.01	0.97	18
TAC/DNA	-0.26	0.46	10	0.23	0.38	17

Reproductive effort was not statistically different among the three treatment groups ($F_{2,23}$ = 0.45, P = 0.64), nor was change in mass from pre- to post-breeding ($F_{2,10}$ = 0.84, P = 0.46).

The model of the change in oxidative damage (protein and DNA combined, PC1 as describe above) from pre- to post-breeding was significant, driven by the additive effects of reproductive effort and treatment group ($F_{3,6}$ = 5.27, P = 0.04, r^2 = 0.73; Fig. 1a, b).



a)

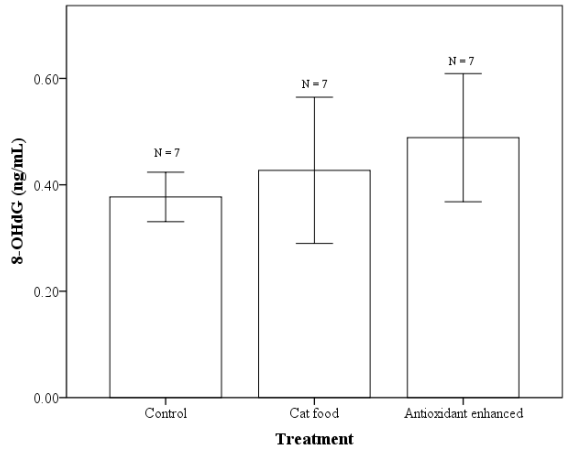


b)

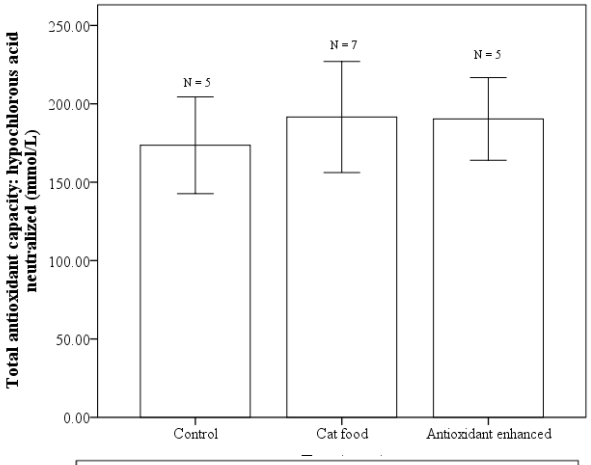
Figure 1: The change in oxidative damage to DNA and proteins (combined with PCA) from pre- to post-breeding in male Florida scrub-jays varied significantly in a model with reproductive effort (a) and treatment (b): $\bar{X} \pm$ SE).

However, neither reproductive effort nor treatment group was significant within the model when considered independently. When analysing only post-breeding measures, there were no treatment effects on oxidative damage to DNA or protein, or in TAC ($F_{2,14}$ = 0.021, P = 0.979; $F_{2,12}$ = 0.523, P = 0.672; and $F_{2,9}$ = 2.925, P = 0.130; respectively; Fig. 2a,b,c).

a)



b)



c)

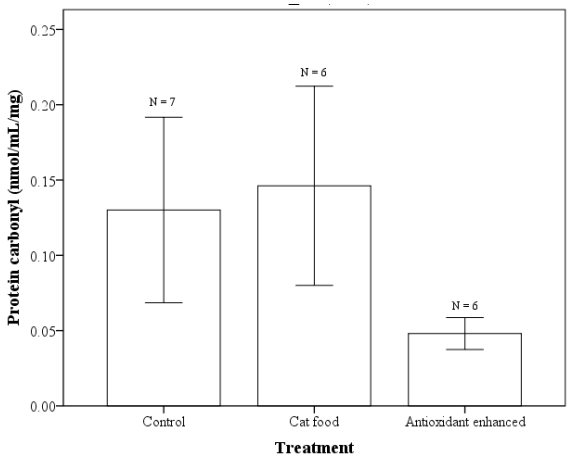


Figure 2: Male Florida scrub-jays demonstrate no effect of treatment on oxidative damage to a) DNA or b) protein, or in c) TAC. Error bars are $\bar{X}$± SE.

There were no significant differences between pre- and post- breeding TAC ($F_{1,5} = 0.45$, $P = 0.53$) or levels of oxidative damage to DNA ($F_{1,8} = 2.43$, $P = 0.158$) in the same model accounting for reproductive effort, change in mass, and treatment. There was however, a significant interaction between change in mass and pre- and post-breeding levels of oxidative damage to proteins (see Table 2).

Table 2. A significant interaction exists between change in protein carbonyl levels from pre- to post- breeding (Time) and change in mass from pre- to post-breeding in male Florida scrub-jays.

Variable	**F**	**P-value**
Time	0.15	0.72
Time* Change in Mass	9.34	0.04
Time* Reproductive Effort	0.06	0.82
Time* Treatment	0.66	0.57

Post-hoc exploration of change in carbonyl and change in mass revealed a strong significant relationship ($F_{1,8}$ = 15.77, P = 0.004; Fig. 3).

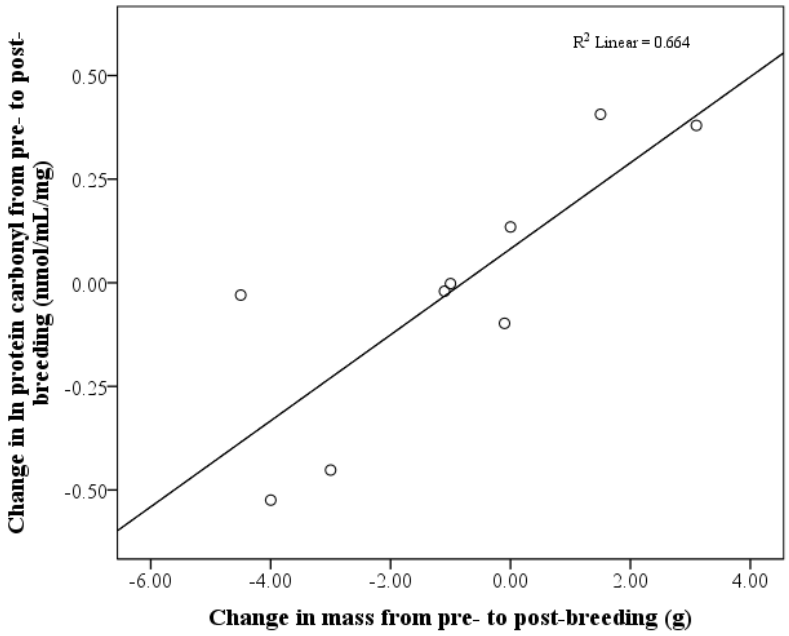


Figure 3: Change in protein carbonyl levels and mass from pre- to post-breeding were significantly related in male Florida scrub-jays.

DISCUSSION

In their review, Metcalfe and Alonso-Alvarez (2010) called attention to the lack of research that had examined the relationship between oxidative state and reproductive effort in *free-living* birds. They further called for consideration of actual measures of damage, (as opposed to proxy measures of oxidative state),

to better document the potential trade-offs between reproductive effort and oxidative balance. Our study addresses both of these concerns and begins to untangle the convoluted relationship between oxidative balance and reproduction in a free-living bird.

One of the most debated topics of avian oxidative stress physiology is the role of carotenoids (Pérez-Rodríguez, 2009). Our results demonstrate that the change in oxidative damage during reproduction varied significantly with both treatment and reproductive effort. Overall damage appears to increase with reproductive effort while controlling for treatment. Further, while firm conclusions are problematic due to low sample sizes, the lower overall oxidative damage of the antioxidant-treated jays is suggestive that these antioxidants confer some degree of protection from damage during reproduction.

Several captive studies of zebra finch have demonstrated a decreased resistance to an oxidative attack after experimental manipulation to increase brood size (Alonso-Alvarez et al., 2004, 2006; Wiersma et al., 2004). A separate study on captive zebra finches found no relationship between resistance to ROS-induced hemolysis (one measure of oxidative stress) and reproductive effort, but treatment with carotenoids significantly reduced the oxidative stress incurred by breeding birds (Bertrand et al., 2006). Our study of a free-living bird demonstrates that both reproductive effort and treatment with antioxidants affect oxidative state. Of particular note is that our combined protein and DNA oxidative damage measure did not increase across the reproductive season in birds

supplemented with antioxidants as it did in the other two treatment groups (see Fig. 1b).

While numerous studies have examined the importance of carotenoids to oxidative balance (for review see Costantini and Møller, 2008), they are characterized by their disparate findings and, to the best of our knowledge, only Hõrak et al. (2010) specifically assessed oxidative *damage.* Further, all were conducted on species that depend on carotenoids for plumage coloration. Here, we suggest that the research on carotenoids as antioxidants needs to be expanded to birds that are not reliant on carotenoid-based secondary sexual traits. The use of model systems, like Florida scrub-jays, in which carotenoids are not used for plumage coloration, allows the elimination of one variable from the complex web of functions that carotenoids may serve. We suggest that carotenoids serve an important antioxidant function in some instances.

We found that changes in mass across the breeding season were positively associated with changes in carbonyl levels. Birds that increased mass during reproduction also suffered the highest levels of oxidative damage to proteins. This may reflect the well-established positive relationship between caloric intake and the accompanying increased metabolism and oxidative stress; in contrast, caloric restriction decreases these indicators (e. g., Sohal and Weindruch, ‘96; Masoro, 2002; Holmes and Ottinger, 2003). Therefore, birds that gained mass during the breeding season may also have paid an oxidative balance cost.

Unexpectedly, we did not find that protein oxidative damage levels of males increased from pre- to post-breeding as they did in the same population of jays the previous year (Heiss and Schoech, *in review*). We suggest that these opposing findings are due to year-effects that resulted from a particularly good overwinter crop of acorns (jays cache acorns extensively and their retrieval is a major source of food for Florida scrub-jays through the winter and early spring months when insect abundance is low; Woolfenden and Fitzpatrick, '96). The 2010 crop of acorns appeared to be among the largest in 20 years of study in this tract (SJS personal observation). It may be that this abundance and the resultant ease with which males could find food, ameliorated the efforts of the 2011 breeding season, such that males avoided the increase in oxidative damage associated with reproduction that was observed in 2010. Our findings highlight the need for studies that encompass measures of oxidative status and environmental conditions across multiple years to better elucidate the relationship between oxidative status and reproductive effort. Clearly, costs associated with reproduction that are readily apparent in a resource poor year may be obscured under conditions in which resources are abundant.

Somewhat surprisingly, there were no correlations between any measures of oxidative stress, during either pre- or post-breeding. This highlights the importance of assessing multiple markers of oxidative state as noted in several studies and reviews (Halliwell, '96, '99; England et al., 2000; Halliwell and Whiteman, 2004; Monaghan et al., 2009). Single measures are insufficient to understand the complexities of the oxidative balance system and oxidative

damage to different biomolecules may not necessarily occur in synchrony. For example, Radák et al. (1997) physically trained rats at high altitudes and found no effect on lipid peroxidation, but oxidation of proteins in the same skeletal muscles increased.

Even if damage to several different types of biomolecules occurs, as might be expected during periods of severe oxidative stress, the extent of the damage and time courses of the removal and repair may be very different among tissue or biomolecule types (Halliwell and Whiteman, 2004). While protein carbonyls and 8-OHdG are more stable than other commonly used measures of oxidative stress (e. g., lipid peroxidation products; see Monaghan et al., 2009 for review), their levels are in flux as DNA breakages are repaired and damaged proteins are broken down and removed by the proteasome system (Davies, 2000). Clearly, such a disassociation between production and repair could lead to a lack of correlation among biomarkers, as has been found in our and one other study (England et al., 2000).

Furthermore, in our study, oxidative damage to both DNA and protein were assessed only in plasma. The underlying assumption, that damage to other tissues would correspond with plasma values, is likely erroneous (see Halliwell, ‘98) and different tissues have been shown to vary in their antioxidant capacity, as well as their susceptibility to oxidative damage (Surai et al., ‘96).

The lack of correlation between oxidative damage measures and TAC is another important finding. Several studies have assessed TAC, without corresponding data on measures of oxidative damage to biomolecules (Bertrand

et al., 2006; Cohen et al., 2008; Bourgeon et al., 2011). While at some level, TAC provides a snapshot of the oxidative state of an individual, there are several factors that remain unaccounted for in any measure of TAC. For instance, as Monaghan et al. (2009) noted, the enzymatic antioxidants that comprise the primary ROS defense within most tissues do not correlate with the circulating antioxidants that contribute to the TAC.

While assessment of multiple measures of oxidative state may reveal seeming disconnects among measures, we believe that it is only by assessing multiple biomarkers that full understanding of the dynamic interplay between ROS, antioxidants, ROS-damaged molecules, and repair mechanisms can be achieved. We hope that future research, almost certainly with captive populations, will elucidate the time course over which oxidative damage occurs to various types of biomolecules. Such research will provide an invaluable framework that might ultimately allow researchers to better select biomarkers that best fit their research questions. We eagerly await developments in the field that will reveal the relationships between oxidative state and the function of carotenoids, particularly in free-living species that do not use carotenoids for plumage coloration. Further, we would hope that future research on free-living species would emphasize the collection of long term data (i. e., measures of oxidative state across multiple years). Clearly, oxidative state might markedly differ with variable environmental conditions within and among years and such variance in oxidative state may well have profound fitness implications.

ACKNOWLEDGEMENTS

We thank all at Archbold Biological Station, especially R. Bowman and H. Swain. Special thanks to T. W. Small and E. S. Bridge for feeders, M. A. Rensel, B. C. Jones, S. E. Bebus, E. K. Elderbrock, R. Hanauer in the field, and J. A. Cole, and L. Neatu (Innovatics Laboratories, Inc) for laboratory and assay assistance. Further thanks to Kemin Industries, Inc for providing gratis supplements.

LITERATURE CITED

Alonso-Alvarez C., S. Bertrand, G. Devevey, J. Prost, B. Faivre, O. Chastel, and G. Sorci. 2006. An experimental manipulation of life-history trajectories and resistance to oxidative stress. Evolution 60:1913-1924.

Alonso-Alvarez C., S. Bertrand, G. Devevey, J. Prost, B. Faivre, and G. Sorci. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecol Lett 7:363-368.

Alonso-Alvarez C., L. Pérez-Rodríguez, J. T. García, J. Viñuela, and R. Mateo. 2010. Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. Physiol Biochem Zool 83:110-118.

Alonso-Alvarez C., L. Pérez-Rodríguez, R. Mateo, O. Chastel, and J. Viñuela. 2008. The oxidation handicap hypothesis and the carotenoid allocation trade-off. J Evol Biol 21:1789–1797.

Beckman K.B., and B.N. Ames. 1998. The free radical theory of aging matures. Physiol Rev 78:547-581.

Bertrand S., C. Alonso-Alvarez, G. Devevey, B. Faivre, J. Prost, and G. Sorci. 2006. Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. Oecologia 147:576-584.

Biard C., P.F. Surai, and A.P. Møller. 2006. Carotenoid availability in diet and phenotype of blue and great tit nestlings. J Exp Biol 209:1004-1015.

Bourgeon S., S. Guindre-Parker, and T.D.Williams. 2011. Effects of sibling competition on growth, oxidative stress, and humoral immunity: a two-year brood-size manipulation. Physiol Biochem Zool 84:429–437.

Cohen A.A., M. Hau, and M. Wikelski. 2008. Stress, metabolism, and antioxidants in two wild passerine bird species. Physiol Biochem Zool 81:436–472.

Casagrande S., G. Dell'Omo, D. Costantini, J. Tagliavini , and T. Groothuis. 2011. Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel: a multi-factorial study. Comp Biochem Physiol A 160:16-26.

Costantini D. 2010. Effects of diet quality on serum oxidative status and body mass in male and female pigeons during reproduction. Comp Biochem Physiol A 156:294-299.

Costantini D., L.Carello, and A. Fanfani. 2010. Relationships among oxidative status, breeding conditions and life-history traits in free-living Great Tits *Parus major* and Common Starlings *Sturnus vulgaris*. Ibis 152:793–802.

Costantini D., S. Casagrande, S. De Filippis, G. Brambilla, A. Fanfani, J. Tagliavini, and G. Dell'Omo. 2006. Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J Comp Physiol B 176:329-337.

Costantini D., C. Coluzza, A. Fanfani, and G. Dell'Omo. 2007. Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). J Comp Physiol B 177:723-731.

Costantini D., and G. Dell'Omo. 2006a. Effects of T-cell-mediated immune response on avian oxidative stress. Comp Biochem Physiol A 145:137-142.

_____. 2006b. Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J Comp Physiol B 176:575-579.

Costantini D. and A.P. Møller. 2008. Carotenoids are minor antioxidants for birds. Funct Ecol 22:367-370.

Dalle-Donne I., R. Rossi, R. Colombo, D. Giustarini, and A. Milzani. 2006. Biomarkers of oxidative damage in human disease. Clin Chem 52:601-623.

Davies K.J.A. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 50:279-289.

Drent R.H., and S. Daan. 1980. The prudent parent: energetic adjustments in avian breeding. Ardea 68:225–252.

England T., E. Beatty, A. Rehman, J. Nourooz-Zadeh , P. Pereira, J. O'Reilly, H. Wiseman, C. Geissler, and B. Halliwell. 2000. The steady-state levels of oxidative DNA damage and of lipid peroxidation (F2-isoprostanes) are not correlated in healthy human subjects. Free Radical Res 32:355-362.

Finkel, T., and N.J. Holbrook. 2000. Oxidants, oxidative stress and the biology of aging. Nature 408:239–247.

Golden T.R., D.A. Hinerfeld, and S. Melov. 2002. Oxidative stress and aging: beyond correlation. Aging Cell 1:117-123.

Halliwell B., and M. Whiteman. 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Brit J Pharmacol 142: 231-55.

Halliwell B. 1996. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radical Res 25:57-74.

Halliwell B. 1998. Can oxidative DNA damage be used as a biomarker of cancer risk in humans? Problems, resolutions and preliminary results from nutritional supplementation studies. Free Radical Res 29:469-486.

Halliwell B. 1999. Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. Nutr Rev 57:104-113.

Halliwell B. Gutteridge J. 1999. Free Radicals in Biology and Medicine. Oxford: Oxford University Press.

Heiss R.S., A.A. Cohen, R. Bowman, R.K. Boughton, E. Bridge, K.J. McGraw and S.J. Schoech. 2011. Circulating carotenoid concentrations are positively correlated with later clutch initiation in Florida Scrub-Jays (*Aphelocoma coerulescens*). J Exp Zool A 315A:101-110.

Heiss R. S. and S. J. Schoech*. In Press*. Oxidative cost of reproduction is sex-specific and correlated with reproductive effort in a cooperatively breeding bird, the Florida Scrub-jay. Physiol Biochem Zool.

Helfenstein F., S. Losdat, A.P. Møller , J.D. Blount, and H. Richner. 2010. Sperm of colourful males are better protected against oxidative stress. Ecol Lett 13:213-222.

Holmes D.J., and M.A. Ottinger. 2003. Birds as long-lived animal models for the study of aging. Exp Gerontol 38:1365–1375.

Hõrak P., E. Sild, U. Soomets, T. Sepp, and K. Kilk. 2010. Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. J Exp Biol 213:2225-2233.

Larcombe S.D., C.A. Tregaskes, J.S.Coffey, A.E. Stevenson, L. Alexander, and K.E. Arnold. 2008. The effects of short-term antioxidant supplementation on oxidative stress and flight performance in adult budgerigars, *Melopsittacus undulatus*. J Exp Biol 211:2859-2864.

Losdot S., F. Helfenstein, B. Gaude, and H. Richner. 2011. Reproductive effort transiently reduces antioxidant capacity in a wild bird. Behav Ecol 22:1218-1226.

Masoro E.J. 2002. Caloric Restriction: A Key to Understanding and Modulating Aging. Amsterdam: Elsevier.

Mateos R., and L. Bravo. 2007. Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). J Sep Sci 30:175-191.

Metcalfe N.B., and C. Alonso-Alvarez. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct Ecol 24:984-996.

Monaghan P., N.B. Metcalfe, and R.Torres. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett 12:75-92.

Mougeot F., J. Martínez-Padilla, L.M.I. Webster, J. D. Blount, L. Pérez-Rodríguez, and S.B. Piertney. 2009. Honest sexual signaling mediated by parasite and testosterone effects on oxidative balance. Proc R Soc B 276:1093-1100.

Nilsson J.A., and L. Råberg. 2001. The resting metabolic cost of egg laying and nestling feeding in great tits. Oecologia 128:187-192.

Nilsson J.A. 2002. Metabolic consequences of hard work. Proc R Soc B 269:1735-1739.

Pérez-Rodríguez L. 2009. Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays 31:1116-1126.

Peterson C.C., K.A. Nagy, and J. Diamond. 1990. Sustained metabolic scope. Proc Nat Acad Sci USA 87:2324-2328.

Radák Z., K. Asano, K. Lee, H. Ohno, A. Nakamura, H. Nakamoto, and S. Goto. 1997. High altitude training increases reactive carbonyl derivatives but not lipid peroxidation in skeletal muscle of rats. Free Rad Biol 22:1109-1114.

Schoech S.J., and R. Bowman. 2003. Does differential access to protein influence differences in timing of breeding of Florida Scrub-Jays (*Aphelocoma coerulescens*) in suburban and wildland habitats? Auk 120:1114-1127.

Schoech S.J., R.L. Mumme, and J.C.Wingfield. 1996. Prolactin and helping behaviour in the cooperatively breeding Florida scrub-jay, *Aphelocoma c. coerulescens*. Anim Behav 52:445-456.

Shen J, P. Deininger, J.D. Hunt, and H. Zhao. 2007. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) as a potential survival biomarker in patients with nonsmall-cell lung cancer. Cancer 109:574-580.

Sohal R.S., and R. Weindruch. 1996. Oxidative stress, caloric restriction, and aging. Science 273:59–63.

Somogyi A., K. Rosta, P. Pusztai, Z. Tulassay, and G. Nagy. 2007. Antioxidant measurements. Physiol Meas 28:R41-R55.

Surai P.F., R.C. Noble, and B.K. Speake. 1996. Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. BBA-Lipid Lipid Met 1304:1-10.

van de Crommenacker J., J. Komdeur, and D.S. Richardson. 2011. Assessing the cost of helping: the roles of body condition and oxidative balance in the Seychelles warbler (*Acrocephalus sechellensis*). PloS One 6:e26423.

Wiersma P., C. Selman, J.R. Speakman, and S.Verhulst. 2004. Birds sacrifice oxidative protection for reproduction. Biol Lett 271:S360-S363.

Wilcoxen T.E, R.K. Boughton, and S.J. Schoech. 2010. Selection on innate immunity and body condition in Florida scrub-jays throughout an epidemic. Biol Lett 6:552-554.

Woolfenden G.E., and J.W. Fitzpatrick. 1984. The Florida Scrub Jay: Demography of a Cooperative-Breeding Bird. Princeton, NJ: Princeton University Press.

Woolfenden G.E., and J.W. Fitzpatrick. 1996. Florida Scrub-Jay (*Aphelocoma coerulescens*), The Birds of North America Online (Poole A., and Gill F. eds.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online.

CHAPTER 4: EXPERIMENTAL SUPPLEMENTATION OF NESTLING FLORIDA SCRUB-JAYS (*APHELOCOMA COERULESCENS*) WITH CAROTENOIDS HAS NO EFFECT ON GROWTH OR OXIDATIVE STATE

Introduction

Recently, there has been considerable interest in how early life conditions, particularly stress, can exert effects throughout the life of an organism (for reviews see Metcalfe and Monaghan, 2001; Schoech et al., 2011). In spite of this, our understanding of the consequences of these early experiences in free-living animals is still limited (Noguera et al., 2011). The developmental period, accompanied by rapid growth, is a demanding and vulnerable time for any organism, and perturbations during this period can result in large effects on survival and reproductive fitness (Lindström, 1999). While Metcalfe and Monaghan (2003) have suggested the that rapid growth of nestling birds may help individuals escape this vulnerable stage earlier and ultimately permit increased survival, a number of studies have found negative impacts of fast growth. These include reduced lifespan, reduced immune capacity, and increased predation risk (reviewed in Arendt, 1997; Blanckenhorn, 2000; Gotthard, 2001; Metcalfe and Monaghan, 2003; Dmitriew, 2010).

Oxidative damage is an additional negative consequence of rapid growth that has gained recent attention (Kim et al., 2011). During events that are energetically demanding, such as growth or reproduction, increased metabolism results in greater production of reactive oxygen species (ROS) that can cause damage to biomolecules if not countered by antioxidant defenses (Davies, 2000;

Nilsson, 2002; Halliwell and Gutteridge, 2007). Recent work in captive zebra finches (*Taeniopygia guttata*) demonstrated this cost. Nestlings that grew faster exhibited lowered red blood cell resistance to free radicals than slower-growing nestlings (Alonzo-Alvarez et al., 2007). As demonstrated by these studies, early growth and development can significantly alter the oxidative balance of an organism. Given that oxidative balance has been proposed to be a major determinant of life history decisions (Monaghan et al., 2009), such alterations at early life stages can have potential long term consequences.

Carotenoids, non-synthesizable pigments with antioxidant properties, have gained attention for their role in mediating oxidative balance (Catoni et al., 2008; Costantini and Møller, 2008). Carotenoids are immune-stimulatory as they increase the cytotoxic, phagocytic, and bacterial-killing abilities of various leukocytes, among other roles (Chew, 1993; Hughes, 2001; Saino et al., 2003; McGraw and Ardia, 2005). Further, carotenoids have been linked to reductions in brain malformation in both mammals and birds (for review see Ramakrishna, 1999). The effects of carotenoids on growth, however, are not as clear. Some studies report that carotenoids have little influence on growth rates (Haq et al., 1995; Royle et al., 1999; Fenoglio et al., 2002), while others have found a positive association between carotenoid supplementation and growth (Tanvez, 2004; Cucco et al., 2006). If there is a trade-off between growth and maintenance of oxidative balance, we anticipate that supplementation with carotenoids would help alleviate the cost of rapid growth by reducing oxidative damage.

This key life-history trade-off between growth and oxidative balance was recently examined in red-winged blackbird (*Agelaius phoeniceus*) nestlings that were supplemented with a mixture of vitamins and minerals (Hall et al., 2010). Nestlings supplemented with antioxidants (not carotenoid specific) exhibited increased growth, but did not differ from controls in levels of lipid peroxidation (one marker of oxidative damage). Another recent study of nestling Eurasian kestrels (*Falco tinnunculus*) found no effect of carotenoid supplementation upon growth or measures of oxidative stress (reactive oxygen metabolites and serum antioxidant capacity; Costantini et al., 2007). While both studies assessed growth and oxidative status, the results are difficult to compare as both studies followed different supplementation regimes and measured different biomarkers of oxidative status.

In the current study, we supplemented free-living Florida Scrub-jay (*Aphelocoma coerulescens*) nestlings with carotenoids and assessed multiple markers of oxidative status. This enabled us to: 1) specifically address the role of carotenoids in the relationship between growth and oxidative balance, and 2) assess correlations among measures of oxidative status in a developing passerine.

Methods

STUDY SPECIES AND SITE

All research was conducted at Archbold Biological Station (ABS), Highlands County, Florida, USA (27°10’N, 81°21’W, elevation 38–68 m; for further details see Schoech, 1996; Schoech and Bowman, 2003).

Florida Scrub-jays are cooperative breeders that live in groups that consist of a pair of socially and genetically monogamous (Quinn et al., 1999; Townsend et al., 2011) breeders and from none to six non-breeding “helpers,” (typically the young of the breeding pair from previous years). These relatively long-lived birds have an extended developmental period during which they are largely reliant upon their parents for provisioning. It is not until approximately 70 days post-hatch that fledglings become nutritionally independent (Woolfenden and Fitzpatrick, 1996).

Nests of breeding pairs in this population are found during the building or laying stages. The average clutch size ranges from 2.9 to 3.7 eggs (Woolfenden and Fitzpatrick, 1996). Nests are continuously monitored and toenails of nestlings are marked at hatching with nail polish to identify hatch order and establish a unique identity. As part of our routine monitoring, at day 11 post-hatch all nestlings are banded with a unique combination of colored plastic leg bands, and a numbered aluminum U.S. Geological Survey band that allow us to track individuals throughout their lifetimes.

SUPPLEMENTATION, GROWTH RATE, AND BLOOD SAMPLING

Beginning at day 3 post-hatch, through day 10 post-hatch, nestlings from seven nests were supplemented once a day between 11-13h with either an oral dose of antioxidants (N = 11 individuals: DSM, FloraGlo 20% #80447, containing 20% lutein and 0.86% zeaxanthin) diluted in 100 µl of water or distilled water (N = 6 individuals). Treatment group was assigned to control for hatch order, such that an equal number of first- and last-hatched nestlings from each nest were in

control or antioxidant-supplemented groups. All nestlings were removed from the nest and supplemented according to their treatment using a micro-pipette. Following the calculations of Biard et al. (2006), carotenoid dosage in the experimental group increased to account for mass changes with growth. For days 3-6, nestlings were supplemented with 24 µg of carotenoids and from days 7-10 the dosage increased to 45 µg. Dosages were based on blood volume estimates based on body mass, efficiency of carotenoid absorption (in birds, generally accepted at 20%; Surai, 2002), and circulating levels of carotenoids present in Florida Scrub-jays (Heiss et al., 2011). Confirmation of effective carotenoid dosage was established in an outside lab (D. Kopsell, Univer. Tenn.) via carotenoid extraction followed by a High Performance Liquid Chromatography (HPLC) assay. Plasma was run in duplicate to assess levels of lutein and zeaxanthin in control and supplemented groups (for detailed extraction and High Performance Liquid Chromatography assay protocol see Wensel et al., 2007; Kopsell et al., 2009). Both lutein and zeaxanthin were higher in carotenoid-supplemented than in control birds (0.88 µl/mL and 0.29 µl/mL, versus 0.48 µl/mL and 0.26 µg/mL, respectively) although likely zeaxanthin was not significantly increased in antioxidant supplemented birds. Unfortunately these changes cannot be explored statistically as plasma for both control and treatment groups had to be pooled in order to secure enough plasma to run the HPLC assay.

Mass of nestlings was measured on days 3-10 prior to supplementation, and tarsus was measured on days 3, 5, and 8. For each individual, daily mass and the mass to tarsus ratios were separately plotted against day with the

resultant slopes retained as measures of growth rate. The slope of the plotted mass against day data will be referred to as “mass accumulation rate” while the mass by tarsus growth rate will be referred to as “body size corrected mass accumulation rate.”

On day 11 post-hatch, all nestlings were removed from their nest and immediately blood sampled. By employing several people for sampling, we assured that each individual was sampled within 120 seconds of nest disturbance, thereby minimizing the possible alteration of blood chemistry values due to the stress of handling. Following brachial venipuncture with a 25-gauge needle, blood samples were collected (~150 µL) in heparinized microhematocrit tubes. Samples were kept chilled with 'cool-packs' (also known as 'Blue-Ice^{TM}') in an insulated bag until return to the laboratory (within 1h) where they were immediately centrifuged to separate the cellular and plasma portions. Plasma was drawn off and stored in sealed vials at -80^{o} C until analyses for oxidative status.

Morphometric data (body mass, length of 7th primary, tarsus, and head-plus-bill) of all nestlings were taken following blood sampling (see Reynolds et al., 2003 for further details). To control for diel fluctuations in physiologic measures, all blood samples were collected between 1100 and 1300.

MEASURES OF OXIDATIVE STATUS

Protein Carbonyl Assay

Protein carbonyls form relatively rapidly in an individual experiencing oxidative stress and, as they are stable, they are a widely used tractable

biomarker of oxidative damage (Monaghan et al., 2009 for review). A single protein carbonyl assay kit (Cayman Chemical Company #10005020) was used for analysis of all samples (intra-assay CV = 7.81%). Briefly, a reaction between protein carbonyls and 2,4-dinitrophenylhydrazine (DNPH) produces a protein-hydrazone that can then be quantified spectrophotometrically (at 360 nm). Because samples likely vary in initial protein concentration and proteins may be lost during the washing steps of the assay, each sample was standardized to the specific protein concentration of the final pellet. These concentrations were quantified spectrophotometrically at 280 nm from a bovine serum albumin standard curve (see Resnick and Packer, 1994, for a detailed protocol).

Total Antioxidant Capacity

The Oxy-Adsorbent test (Diacron International, Grosseto, Italy) provided a quantitative measure of total antioxidant capacity (TAC) by evaluating the ability of plasma to withstand a controlled free radical attack by hypochlorous acid (HClO, a biologically relevant oxidant: Costantini et al., 2006; Costantini and Dell'Omo, 2006a,b). Briefly, 10 µL of plasma were diluted 1:100 with dH_2O, and the diluted plasma was incubated for 10 minutes at 37^oC with 200 µL of a HClO solution, following which 5 µL of N, N-diethyl-p-phenylenediamine (a chromogen) were added. Immediately thereafter, absorbance was read at 505 nm and compared with standards. The antioxidant capacity is expressed as micromoles of neutralized HClO per mL of sample. It is important to note that while other methods of measuring plasma antioxidant capacity have been criticized for their overemphasis of the contributions of uric acid, the contribution of uric acid to TAC

as measured by this assay is low (Costantini, 2010). A single kit was used for this analysis (intra-assay CV = 3.90%).

Reactive Oxygen Metabolites (ROMs)

Hydroperoxides, compounds that signal oxidative tissue damage to lipids and proteins, were measured via a d-ROMs assay kit (Diacron International, Grosseto, Italy). We diluted 4 µl of plasma, calibrator, or distilled water (blank) in 202 µl of the provided acidic buffer solution in duplicate, onto a 96-well plate. The plate was mixed gently and incubated for 75 min at 37^{o}C. Immediately following incubation, absorbance was measured at 505 nm and ROM concentration was calculated in millimolar of H_2O_2 equivalents (as per Haussmann et al., 2012). All samples were run in a single assay with an intra-assay CV of 14.6%. The ratio of ROMs: TAC * 1000 was calculated to represent plasma oxidative status, with high values reflective of high oxidative stress levels (Casagrande et al., 2011).

STATISTICAL ANALYSES

Differences in growth rate between supplemented and nonsupplemented nestlings were assessed in a series of General Linear Models. Day 11 mass, mass accumulation rate, and body size corrected mass accumulation rate were assessed individually (i. e., were dependent variables in separate models), with hatch order and treatment group as factors.

We then assessed correlations among the measures of oxidative state in a Pearson's correlation matrix which included all measures of oxidative status (protein carbonyl, TAC, ROMs, and plasma oxidative status).

Finally, we explored differences in oxidative status between supplemented and nonsupplemented nestlings, and interactions between growth rate and antioxidant status in a second series of General Linear Models. Protein carbonyl, TAC, ROMs, and plasma oxidative status were investigated separately, with hatch order and treatment group included in the model as factors and growth rate measures as covariates.

Results

None of the measures of oxidative state were correlated (see Table 1).

Table 1. Measures of oxidative state in nestling Florida Scrub-jays are not correlated with one another (N =17).Please note that Plasma Oxidative Status is not analyzed with measures of dROMs or TAC as it is derived from these measures.

	Pearson's r	**P-value**
Protein carbonyl/TAC	0.02	0.94
Protein carbonyl/dROMs	- 0.21	0.43
TAC/dROMs	0.25	0.34
Plasma Oxidative Status/ Protein Carbonyl	- 0.21	0.42

We found no differences in day 11 mass, nor either measure of growth rate between carotenoid supplemented and control nestlings. Day 11 mass, mass accumulation rate, and body size corrected mass accumulation rate, were all statistically indistinguishable among birds in the different treatment groups ($F_{5,11}$ = 2.70, P = 0.08; $F_{5,11}$ = 1.88, P = 0.18; $F_{5,11}$ = 2.98, P = 0.06, respectively). Post-hoc exploration of the trend for differences between treatment groups using day 11 mass was further explored by taking the residuals of day 11 mass for hatch order with treatment removed from the GLM. When controlling for hatch order in this manner, the trend was for antioxidant supplemented birds to be larger than their control counterparts ($\bar{X}$ ± SE: N = 11, 1.14 ± 5.13 versus N = 6, -2.09 ± 6.20, respectively). Similarly, post-hoc exploration of the trend for differences between treatment groups using body size corrected mass accumulation rate was explored as above by taking the residuals for hatch order with treatment removed from the GLM. When controlling for hatch order in this matter, the trend was for antioxidant supplemented birds to be smaller than their control counterparts ($\bar{X}$ ± SE: N = 11, -0.029 ± 0.098 versus N = 6, 0.053 ± 0.084, respectively). The post-hoc explorations of these trend is indicative that antioxidant supplemented birds may have been able to increase overall mass, but did not appear to be increasing relative body size any faster than their control counterparts.

We also found no relationships among treatment, measures of growth, and any measure of oxidative state (protein carbonyl: $F_{8,17}$ = 1.92, P = 0.13; TAC:

$F_{8,17}$ = 1.50, P = 0.23; ROMs: $F_{8,17}$ = 1.93, P = 0.13; plasma oxidative status: $F_{8,17}$ = 1.82, P = 0.14).

Discussion

We found no relationships between growth and oxidative status in Florida Scrub-jay nestlings. Further, despite our treatment which increased nestling plasma levels of carotenoids (at least in levels of lutein), our supplementation had no effect on their rates of growth, overall mass, or any measure of oxidative state.

Evidence for the role of carotenoids in facilitating rapid early growth is inconclusive. While some studies note a positive effect on nestling growth (e. g., Tanvez, 2004; Cucco et al., 2006; Hall et al., 2010), others found no link between carotenoid supplementation and early life growth patterns (Haq et al., 1995; Royle et al., 1999; Fenoglio et al., 2002; Saino et al., 2003).

Despite our treatment which delivered increased lutein antioxidants to selected nestlings, we found no effect on overall oxidative status. To our knowledge, this and one other (Costantini et al., 2007), are the only studies that have addressed the oxidative response of nestling birds to carotenoid-supplementation. Because carotenoid levels in Florida Scrub-jays are naturally lower than those of Eurasian kestrels (Constantini et al., 2007) and because carotenoid pigments are not used for coloration in scrub-jays, we expected that carotenoids would be more dedicated to their roles as antioxidants in scrub-jays. However, our results paralleled those of Costantini et al. (2007) in that supplemental carotenoids did not affect measures of growth or oxidative state.

The specific finding that TAC was not increased by supplementation was particularly surprising, given the increased levels of circulating carotenoids in the plasma of supplemented nestlings. While it is possible that carotenoids do not make large contributions to TAC (see Costantini and Møller, 2008), it has been widely demonstrated that HOCl reacts with carotenoids (Handelman et al., 1991; Siems et al., 2000) and therefore we do not expect that our lack of significant differences between treatment groups is method based.

While the immediate post-hatching period is a crucial time for birds in the maintenance of oxidative balance (Freeman and Vince, 1974; Vleck and Bucher, 1998), it is possible that maternally transferred carotenoids are more important for protection against damage during this time period (see Blount et al., 2002a, b), than those carotenoids that are acquired via diet, (e. g. our supplemental source). Further, a wide range of factors may influence the absorption of carotenoids; from the lipid content of recently consumed food to physiological constraints in the gut (see Casagrande et al., 2007). Differences among individuals may have limited the availability and effectiveness of our supplemental carotenoids to protect against oxidative damage.

Another possibility to explain the apparent lack of antioxidant activity by the supplemental carotenoids is that there may have been an absence (or very low levels) of other antioxidants (e.g., vitamin E or C), thus preventing synergistic antioxidant activity (Pérez-Rodríguez, 2009). Finally, our finding that measures of oxidative state were not correlated highlights the importance of the use of multiple indices for oxidative status; a recommendation we have stressed in

previous work that reports a similar lack of correlation between measures of oxidative state (Heiss and Schoech, *in review*).

Acknowledgements

We thank all at Archbold Biological Station, especially R. Bowman and H. Swain. Special thanks to T. W. Small, M. A. Rensel, B. C. Jones, S. E. Bebus, E. K. Elderbrock, and R. Hanauer. Also, a hearty “thank you” to D.E. Kopsell. This research was funded by a National Science Foundation collaborative grant to S.J. Schoech and E.S. Bridge (IOS-0919899 and -0919528) as well as a National Science Foundation Doctoral Dissertation Improvement Grant to R. S. Heiss and S. J. Schoech (IOS-1110315). All work was conducted under federal and state permits to S. J. Schoech (TE117769-3 and WX08091A, respectively) and University of Memphis IACUC approval to R. S. Heiss (#0667).

References

Alonso-Alvarez, C., Bertrand, S., Faivre, B. and Sorci, G. (2007). Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. *Funct. Ecol.* **21**, 873-879.

Arendt, J. D. (1997). Adaptive intrinsic growth rates: an integration across taxa. *Q. Rev. Biol*. **72**, 149–177.

Biard, C., Surai, P. F. and Møller, A. P. (2006). Carotenoid availability in diet and phenotype of blue and great tit nestlings. *J. Exp. Biol*. **209**, 1004-1015.

Blanckenhorn, W. U. (2000). The evolution of body size: what keeps organisms small? *Q. Rev. Biol.* **75**, 385–407.

Blount, J. Surai, P. F. and Houston, D. C. (2002). Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Funct. Ecol.* **16**, 445-453.

Blount, J., Surai, P. F. and Nager, R. G. (2002). Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proc. R. Soc. B*. **269**, 29-36.

Casagrande, S., Costantini, D., Fanfani, A., Tagliavini, J. and Dell'Omo, G. (2007). Patterns of serum carotenoid accumulation and skin colour variation in kestrel nestlings in relation to breeding conditions and different terms of carotenoid supplementation. *J. Comp. Physiol. B.* **177**, 237–245.

Casagrande, S., Dell'omo, G., Costantini, D., Tagliavini, J. and Groothuis, T. (2011). Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel: a multi-factorial study. *Comp. Biochem. Physiol. A.* **160**, 16–26.

Catoni, C., Peters, A. and Martinschaefer, H. (2008). Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. *Anim. Behav.* **76**, 1107-1119.

Chew, B. P. (1993). Role of carotenoids in the immune-response. *J. Dairy Sci*. **76**, 2804–2811.

Costantini, D. (2010). Effects of diet quality on serum oxidative status and body mass in male and female pigeons during reproduction. *Comp. Biochem. Physiol. A*. **156,** 294-299.

Costantini, D. and Dell'Omo, G. (2006a). Effects of T-cell-mediated immune response on avian oxidative stress. *Comp. Biochem. Physiol. A.* **145**, 137–142.

Costantini, D. and Dell'Omo, G. (2006b). Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *J. Comp. Physiol. B*. **176**, 575–579.

Costantini, D., and Møller, A. P. (2008). Carotenoids are minor antioxidants for birds. *Funct. Ecol*. **22**, 367–370.

Costantini, D., Casagrande, S., De Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J. and Dell'Omo, G. (2006). Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *J. Comp. Physiol. B.* **176**, 329-337.

Costantini, D., Fanfani, A. and Dell'Omo, G. (2007). Carotenoid availability does not limit the capability of nestling kestrels (*Falco tinnunculus*) to cope with oxidative stress. *J. Exp. Biol.* **210**, 1238-1244.

Costantini, D., Marasco, V. and Møller, A.P. (2011). A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. *J. Comp. Physiol. B.* **181**, 447-456.

Cucco, M., Malacarne, G., Ottonelli, R. and Patrone, M. (2006). Repeatability of cell-mediated and innate immunity, and other fitness-related traits, in the grey partridge. *Can. J. Zool*. **84**, 72–79.

Davies, K. J. A. (2000). Oxidative stress, antioxidant defenses, and damage removal, repair and replacement systems. *IUBMB Life*, **50**, 279-289.

Dmitriew, C. M. (2010). The evolution of growth trajectories: what limits growth rate? *Biol. Rev*. **86**, 97-116.

Fenoglio, S., Cucco, M. and Malacarne, G. (2002). The effect of a carotenoid-rich diet on immunocompetence and behavioural performances in Moorhen chicks. *Ethol. Ecol. Evol.* **14**, 149–156.

Freeman, B. M. and Vince, M. A. (1974). Development of the Avian Embryo. New York: John Wiley.

Gotthard, K. (2001). Growth strategies of ectothermic animals in temperate environments. In: *Environment and animal development: genes, life histories and plasticity* (eds. D. Atkinson and M. Thorndike), pp. 287-303. Oxford: BIOS Scientific Publishers.

Hall, M. E., Blount, J. D., Forbes, S. and Royle, N. J. (2010). Does oxidative stress mediate the trade-off between growth and self-maintenance in structured families? *Funct. Ecol.* **24**, 365-373.

Halliwell, B. and Gutteridge, J. (2007). Free Radicals in Biology and Medicine. Oxford: Oxford University Press.

Handelman, G. J., van Kuijk, F. J., Chatterjee, A. and Krinsky, N. I. (1991). Characterization of products formed during the autoxidation of β-carotene. *Free Radic. Biol. Med*. **10**, 427-437.

Haq, A. U., Bailey, C. A. and Chinnah, A. (1995). Neonatal immune response and growth performance of chicks hatched from single comb White Leghorn breeders fed diets supplemented with beta-carotene, canthaxanthin, or lutein. *Poult. Sci.* **74**, 844–851.

Haussmann, M. F., Longenecker, A. S., Marchetto, N. M., Juliano, S. A. and Bowden, R. M. (2012). Embryonic exposure to corticosterone modifies the juvenile stress response, oxidative stress and telomere length. *Proc. R. Soc. B*. **279**, 1447-1456.

Heiss, R. S., Cohen, A. A., Bowman, R., Boughton, R. K., Bridge, E., McGraw, K. J. and Schoech, S. J. (2011). Circulating carotenoid concentrations are positively correlated with later clutch initiation in Florida Scrub-Jays (*Aphelocoma coerulescens*). *J. Exp. Zool. A.* **315A**, 101-110.

Hughes, D. A. (2001). Dietary carotenoids and human immune function. *Nutrition* **17**, 823–827.

Kim, S.-Y., Noguera, J. C., Morales, J. and Velando, A. (2011). Quantitative genetic evidence for trade-off between growth and resistance to oxidative stress in a wild bird. *Evol. Ecol*. **25**, 461–472.

Kopsell, D.A., Armel, G.R., Mueller, T. C., Sams, C.E., Deyton, D. E., McElroy, J. S. and Kopsell, D. E. (2009). Increase in nutritionally important sweet corn kernel carotenoids following mesotrione and atrazine applications. *J. Agric. Food. Chem.* **57**, 6362-6368.

Lindström, J. (1999). Early development and fitness in birds and mammals. *Trends Ecol. Evol*. **14**, 343–348.

McGraw, K. J. and Ardia, D. R. (2005). Sex differences in carotenoid status and immune performance in zebra finches. *Evol. Ecol. Res.* **7**, 251–262.

Metcalfe, N. B. and Monaghan, P. (2001). Compensation for a bad start: grow now, pay later? *Trends Ecol. Evol.* **16**, 254–260.

Metcalfe, N. B. and Monaghan, P. (2003). Growth versus lifespan: perspectives from evolutionary ecology. *Exp. Gerontol.* **38**, 935-940.

Monaghan, P., Metcalfe, N.B. and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75-92.

Nilsson, J.- Å. (2002). Metabolic consequences of hard work. *Proc. R. Soc. B*. **269**, 1735-1739.

Noguera, J.C., Kim, S.-Y. and Velando, A. (2011). Pre-fledgling oxidative damage predicts recruitment in a long-lived bird. *Biol. Lett*. **8**, 61-63.

Pérez-Rodríguez, L. (2009). Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. *BioEssays*. **31**, 1116–1126.

Quinn, J. S., Woolfenden, G. E., Fitzpatrick, J. W. and White, B. N. (1999). Multi-locus DNA fingerprinting supports genetic monogramy in Florida scrub-jays. *Behav. Ecol. Sociobiol*. **45,** 1-10.

Ramakrishna, T. (1999). Vitamins and brain development. *Physiol. Res.* **48,** 175–187.

Reynolds, S. J., Schoech, S. J. and Bowman, R. (2003). Diet quality during pre-laying and nestling periods influences growth and survival of Florida scrub-jay (*Aphelocoma coerulescens*) chicks. *J. Zool*. **261**, 217–226.

Resnick, A. Z. and Packer, L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Meth. Enzymol*. **233**, 357-363.

Royle, N. J., Surai, P. F., McCartney, R. J. and Speake, B. K. (1999). Parental investment and egg yolk lipid composition in gulls. *Funct. Ecol.* **13**, 298–306.

Sahin, K., Kucuk, O., Sahin, N. and Sari, M. (2002). Effects of vitamin C and vitamin E on lipid peroxidation status, some serum hormone, metabolite, and mineral concentrations of Japanese quails reared under heat stress (34°C). *Int. J. Vitam. Nutr. Res*. **72**, 91-100.

Saino, N., Ferrari, R. P., Romano, M., Martinelli, R. and Møller, A. P. (2003). Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. *Proc. R. Soc. Lond. B.* **270**, 2485–2489.

Schoech S. J. and Bowman, R. (2003). Does differential access to protein influence differences in timing of breeding of Florida Scrub-Jays (*Aphelocoma coerulescens*) in suburban and wildland habitats? *Auk* **120**, 1114-1127.

Schoech, S. J. (1996). The effect of supplemental food on body condition and the timing of reproduction in a cooperative breeder, the Florida Scrub-Jay. *Condor* **98,** 234-244.

Schoech, S. J., Rensel, M. A. and Heiss, R. S. (2011). Short- and long-term effects of developmental corticosterone exposure on avian physiology, behavioral phenotype, cognition, and fitness: A review. *Curr. Zool.* **57,** 514-530.

Siems, W. G., Sommerburg, O., Hurst, J. S. and van Kuijk, F. J. G. M. (2000). Carotenoid oxidative degradation products inhibit Na^+-K^+-ATPase. *Free Radic. Res.* **33**, 427-435.

Surai, P. F. (2002). Natural Antioxidants in Avian Nutrition and Reproduction. Nottingham: Nottingham University Press.

Tanvez, A. (2004). *Effets maternels et qualité de l'oeuf chez le canari domestique commun et le goéland leucophée.* Thèse de doctorat. Paris: Paris X Nanterre.

Townsend A. K., Bowman, R., Fitzpatrick, J. W., Dent, M. and Lovette, I. J. (2011). Genetic monogamy across variable demographic landscapes in cooperatively breeding Florida scrub-jays. *Behav. Ecol*. **22**, 464-470.

Vleck, C. M. and Bucher, T. L. (1998). Energy metabolism, gas exchange, and ventilation. In: *Avian Growth and Development: Evolution Within the Altricial-Precocial Spectrum* (eds. J. M. Starck and R. E. Ricklefs), pp. 89-116. Oxford: Oxford University Press.

Wenzel, A. J. Sheehan, J. P., Burke, J. D., Lefsrud, M. G. and Curran-Celentano, J. (2007). Dietary intake and serum concentrations of lutein and zeaxanthin, but not macular pigment optical density, are related in spouses. *Nutrit. Res.* **27**, 462-469.

Woolfenden, G. E. and Fitzpatrick, J. W. (1996). Florida Scrub-Jay (*Aphelocoma coerulescens*), The Birds of North America Online (eds. A. Poole and F. Gill). Ithaca: Cornell Lab of Ornithology.

CHAPTER 5: AN EXPLORATION OF OXIDATIVE DAMAGE, AGE, AND SURVIVAL IN THE FLORIDA SCRUB-JAY (*APHELOCOMA COERULESCENS*)

INTRODUCTION

Oxidative damage to biomolecules results from the inability of an organism to cope with the actions of reactive oxygen species (ROS), chemically reactive molecules that are formed as byproducts of metabolism. The accrual of damage by ROS has long been at the center of one of the most widely accepted models for aging; the free-radical theory of aging (see Beckman and Ames 1998). Briefly, this theory states that the accumulation of damage to various types of biomolecules with age leads to degradation of function that ultimately results in death (Harman 1956). Over the past decade, the oxidative stress theory of aging has been supported with mounting evidence linking oxidative damage to the aging process (for review see Kregel and Zhang 2007). Several studies on a variety of organisms have documented an accumulation of oxidative damage to biomolecules (e. g., protein, lipid, and DNA) in multiple tissues as organisms age (Sohal et al. 1993, Forster et al. 1996, Yan and Sohal 1998, Yasuda et al. 1999, Hamilton et al. 2001, Stadtman 2001, Takabayashi 2004). However, relatively little research has addressed mechanisms by which organisms repair or eliminate oxidized biomolecules; thereby ameliorating associated disease and aging effects attributable to the damaged molecules (Sohal et al. 2002). Most of the research that has examined repair has focused on DNA repair and the

associated extension of lifespan (Newton et al. 1989 a,b, Starke-Reed and Oliver 1989, Whitehead and Grigliatti 1993, Gredilla et al. 2010, Park et al. 2011). Such repair mechanisms may be of particular importance to longer-lived species.

Oxidized protein accumulation is a particularly good hallmark of aging as protein carbonylation is both irreversible and correlated with oxidative stress-induced cell death (Berlett and Stadtmand 1997, Friguet et al. 2000, Stadtman and Levine 2000, Friguet 2002, Costa et al. 2007). The buildup of oxidized proteins with age has been hypothesized to result from increased protein oxidative damage, decreased oxidized protein degradation, decreased oxidized protein re-building, or a combination of these processes (Frigeuet 2006). The detrimental effects of oxidized proteins include mitochondrial damage leading to enhanced production of ROS, an effect that has been shown to have short-term effects on survival (Herbert 1994, Herbert et al. 1994). Further, oxidative damage to proteasomes, intracellular protein complexes that degrade damaged proteins, can result in increased cellular damage perpetuating a cycle of increasing damage (Sohal et al. 2002). Regardless of the mechanism, the accumulation of toxic carbonyls has been linked to both the commencement and continuation of a variety of diseases, as well as the general aging process (Petropoulos and Friguet 2005).

Antioxidants provide one mechanism for protection against oxidative damage (Mayo et al. 2003). As such, the total antioxidant capacity (TAC) assay has become a popular measure of an organism's ability to resist attack by ROS. Two recent studies in relatively short-lived passerines (the Collared Flycatcher,

Ficedula albicollis, and the Barn Swallow, *Hirundo rustica*) found no variation in TAC with age (Marko et al. 2011, Saino et al. 2011). However, in a long-lived species, the Greater Flamingo (*Phoenicopterus ruber roseus*), resistance to oxidative stress was highest at intermediate ages (Devevey et al. 2009), suggesting that TAC is important in longer-lived species.

Our study population of Florida Scrub-jays (*Aphelocoma coerulescens)* has been studied extensively for 25 years. This is a relatively long-lived species with a maximum lifespan of 15.5 years (Woolfenden and Fitzpatrick 1996), and at any one time, 10–20% of breeding individuals in our population are approximately 10 years of age or older. Recent evidence indicates that older birds in this population do not undergo actuarial senescence (Wilcoxen et al. 2010a, but see McDonald *et al.* 1996). Instead, Wilcoxen et al. (2010 a,b) suggest that those individuals that survive to older ages are high quality birds, whereas those of lower quality have been culled from the population.

The goals of the current study were threefold - to determine whether: 1) oxidized damage to proteins and DNA accumulate with age as predicted by the free radical theory of aging; 2) there was a relationship between age and TAC; and 3) accumulation of oxidative damage or TAC status at an early age, was predictive of survival over the short-term (i. e., the first year of life).

METHODS

Study species and site

Research was conducted at Archbold Biological Station (ABS), Highlands County, Florida, USA (27°10'N, 81°21'W, elevation 38–68 m; for further details

see Schoech 1996, Schoech and Bowman 2003). Because Florida Scrub-jays are a non-migratory, highly sedentary species, virtually all individuals spend their entire lives at ABS. Each scrub-jay in this population is uniquely marked with colored plastic leg bands and a numbered aluminum U.S. Geological Survey band that allow us to track all individuals throughout their lifetimes.

We wished to address any differences in oxidative damage levels during early life stages (nestling, ~70 days old, and 9 months of age), as these are times of high mortality for this species. Woolfenden and Fitzpatrick (1996) note that the mortality rates of Florida Scrub-jays from fledging to ~70 days-of-age, (when they become nutritionally independent from their parents) is 41%. From nutritional independence to 1 year-of-age, mortality is 45%, whereas for each year after year one, annual mortality rates decrease to ~25 ± 5% (depending on sex and breeding status). It may be that the ability to regulate oxidative balance during these early vulnerable stages is of particular importance to survival.

Trapping and blood sample collection

To obtain blood samples, birds were trapped in continually monitored Potter or drop traps from late January through late February of 2010 and 2011. Nutritionally independent young (~70 days post hatch) from both 2010 and 2011 were trapped in early August of their hatch year. Nestlings from 2010 and 2011 were removed from their nest at day 11 post-hatch for blood sampling. Following brachial venipuncture with a 25-gauge needle, blood samples were collected (~150 µL) in microhematocrit tubes within 2 min of capture or removal from the nest, thereby minimizing the alteration of blood chemistry values due to the

stress of capture and handling. Blood samples were kept chilled with 'cool-packs' (also known as 'Blue-Ice^{TM}') in an insulated bag until return to the laboratory (within 1-3 h) where samples were centrifuged to separate the cellular and plasma portions. Plasma was drawn off and stored in sealed vials at -80^{o} C until analysis for TAC and oxidative damage levels.

Morphometric data (body mass, head breadth, distance from the nares-to-bill-tip, and lengths of the wing-cord, head-plus-bill, culmen, and tail) were taken following blood sample collection (see Schoech 1996 for further details). To control for diel fluctuations in physiologic measures, all blood samples were collected between 0700 and 1100.

Measures of oxidative status

Protein carbonyl data were collected for all nestlings, independent young, 9 month old, and adult birds in this study. Because of blood volume limitations, damage to DNA was only measured in adult birds (1 year and older). TAC was measured in adult birds, and 2011 nestlings.

Protein Carbonyl Assay

Protein carbonyls form relatively rapidly in an individual experiencing oxidative stress and, as they are stable, they are a widely used, tractable biomarker of oxidative damage (Monaghan et al. 2009 for review). Nine protein carbonyl assay kits (Cayman Chemical Company #10005020) were used to assay all of the 2010 samples (intra-assay CVs 1.3-4.3%) and ten kits were used to assay samples collected in 2011 (intra-assay CVs 3.5-6.7%; Cayman notes that inter-assay CV is 8.5%). Briefly, a reaction between protein carbonyls and

2,4-dinitrophenylhydrazine (DNPH) produces a protein-hydrazone that can then be quantified spectrophotometrically (at 360 nm). Because samples likely vary in initial protein concentration and proteins may be lost during the washing steps of the assay, each sample was standardized to the specific protein concentration of the final pellet. These concentrations were quantified spectrophotometrically at 280 nm from a bovine serum albumin standard curve (see Resnick and Packer 1994, for a detailed protocol).

Oxidative Damage to DNA

We used Cell BioLabs, Inc; OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation) STA-320, (Shen et al. 2007), to measure the oxidative damage to DNA via the concentration of the oxidative derivative of guanine (8-hydroxy-2'-deoxyguanosine [8-OHdG]) present in plasma. Briefly, 50 µL of plasma (or standard) were added to an 8-OHdG conjugate-coated plate, and incubated at room temperature for 10 minutes. An anti-8-OHdG monoclonal antibody was added, followed by addition of a diluted secondary antibody-enzyme conjugate. The plate was incubated for one hour and triple washed. The absorbance of each micro-well was then determined at 450 nm. A total of three assay kits were used (intra-assay CVs based on standards: 3.5-6.9%, inter-assay CV = 9.0%).

Total Antioxidant Capacity

The Oxy-Adsorbent test (Diacron International, Grosseto, Italy) provided a quantitative measure of total antioxidant defense by evaluating the ability of plasma to withstand a controlled free radical attack by hypochlorous acid (HClO;

a biologically relevant oxidant; Costantini and Dell'Omo 2006a,b, Costantini et al. 2006). Briefly, 10 µL of plasma were diluted 1:100 with dH_2O, and the diluted plasma was incubated for 10 minutes at 37^{o}C with 200µL of a HClO solution, following which 5 µL of N, N-diethyl-p-phenylenediamine (a chromogen) were added. Immediately thereafter, absorbance was read at 505 nm and compared with standards. The antioxidant capacity is expressed as micromoles of neutralized HClO per mL of sample. It is important to note that while other methods of measuring plasma antioxidant capacity have been criticized for their overemphasis of the contributions of uric acid, the contribution of uric acid to TAC as measured by this assay is low (Costantini 2010). A total of six assay kits were used (intra-assay CVs based on standards: 7.1-10.6%, inter-assay CV = 13.0%).

Statistical analyses

To determine that there were no year differences, oxidative damage levels for adult birds in 2010 and 2011 were first analyzed with age as an independent variable, and year as a random factor in a General Linear Model (GLM) to determine if slopes between years were significantly different. Additionally, day of year that individual birds were trapped in 2010 and 2011 was also analyzed in a regression to ensure that the trap date was not influential given that birds were trapped over a period of two months. Because neither day of year, nor year were significant factors in any model (P = 0.14 – 0.58), 2010 and 2011 protein carbonyl data were combined for further analysis of samples from adult birds. A curve estimation regression was used to assess whether age was predictive of carbonyl level. Protein carbonyl data from 2010 and 2011 included twelve birds

that were sampled twice, once in each year. Only the 2010 samples from these birds were incorporated into the analysis to avoid violation of the assumption of independence. A single outlier was removed from the analysis. Although this older bird was characterized by relatively high levels of oxidative damage for its age, we believe this to be a case in which the bird was experiencing distress (possibly disease) given that despite having been a breeder for some years, during the 2011 season he was usurped from his territory and is now missing and presumed dead. Because displacement of breeders occurs rarely, we felt justified that there was a biological basis which justified the removal of this outlier, however, we report results with this bird both included and removed. Curve estimation regressions were also used to assess the relationships between age and both oxidative damage to DNA and TAC.

Prior to conducting analyses on protein oxidative damage levels for 2010 and 2011 nestlings and independent young, these data were first analyzed in separate GLMs with year as a random factor to determine if slopes between years were significantly different. Because year was a significant factor in both the models for nestlings and independent young ($P < 0.001$), data from 2010 and 2011 were analyzed separately. Nestling and independent young carbonyl content were significantly higher in 2011 relative to 2010 ($\overline{X}$ ± SE: 2010; nestlings 0.04 ± 0.00, independent young 2.36 ± 0.53 and in 2011; nestlings, 0.96 ± 0.22, independent young 37.9 ± 7.21).

We used a Linear Mixed Model to determine whether protein carbonyl levels differed among developmental stages (nestling, independent young, and 9

months of age), controlling for repeated sampling and nest of origin. Data are presented as $\bar{X}$ ± SE.

To determine whether carbonyl levels or TAC were predictive of survival, we used a binomial logistic regression with nestling carbonyl level or TAC as the covariate, and survival to nutritional independence (binary) as the dependent variable. Similarly, we assessed survival to 9 months, and one year for both 2010 and 2011, using carbonyl levels of nutritionally independent birds as the covariate. In a final series of binomial logistic regressions, we analyzed the change in carbonyl level (from nestling to nutritional independence, and from independence to 9 months) as predictors of survival to independence and to 9 months, respectively. Unfortunately, survivorship of 2011 birds was too low to assess beyond nutritional independence. All statistical analyses were performed using PASW® Statistics 18.

RESULTS

Age and oxidative state

Oxidative damage to proteins was negatively correlated with age (logarithmic model, outlier removed: $F = 5.04$, df = 1 and 99, $r^2 = 0.05$, $P = 0.03$, outlier included: $F = 3.79$, df = 1 and 100, $r^2 = 0.03$, $P = 0.05$: Fig 1a).

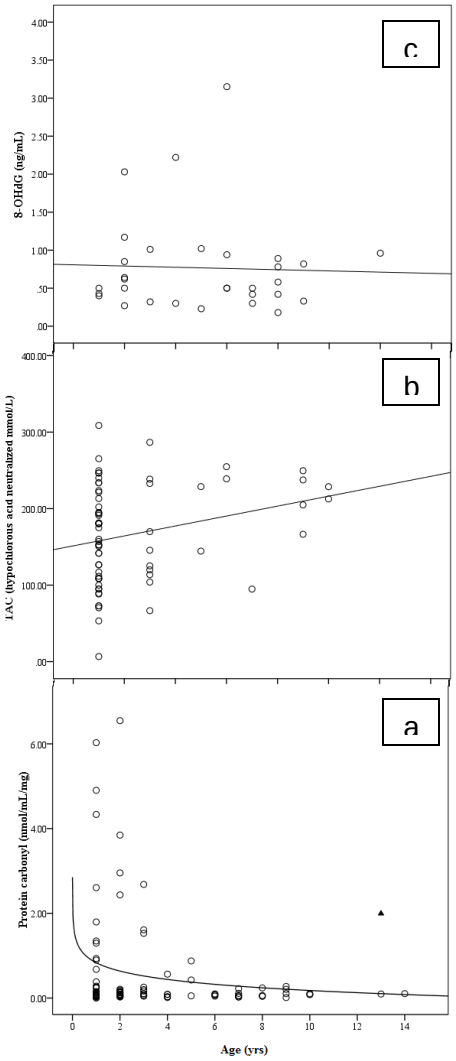


Figure 1: Florida scrub-jays demonstrate a) a negative relationship between age and oxidative damage to proteins, b) a positive relationship between TAC and age and c) do not differ significantly in damage to DNA. Outlier point in 1a is shown as (▲).

We found a positive relationship between age and TAC (linear model: $F = 4.61$, df = 1 and 65, $r^2 = 0.07$, $P = 0.04$: Fig 1b). Age and damage to DNA were not statistically related (linear model: $F = 0.04$, df = 1 and 30, $r^2 = 0.001$, $P = 0.84$: Fig 1c).

Developmental stage and oxidative state

Protein carbonyl levels differed significantly among developmental stages in 2010 ($F = 10.17$, df = 2 and 18, $P = 0.001$: Fig. 2a) and 2011 ($F = 56.79$, df = 1 and 12, $P < 0.0001$: Fig. 2b).

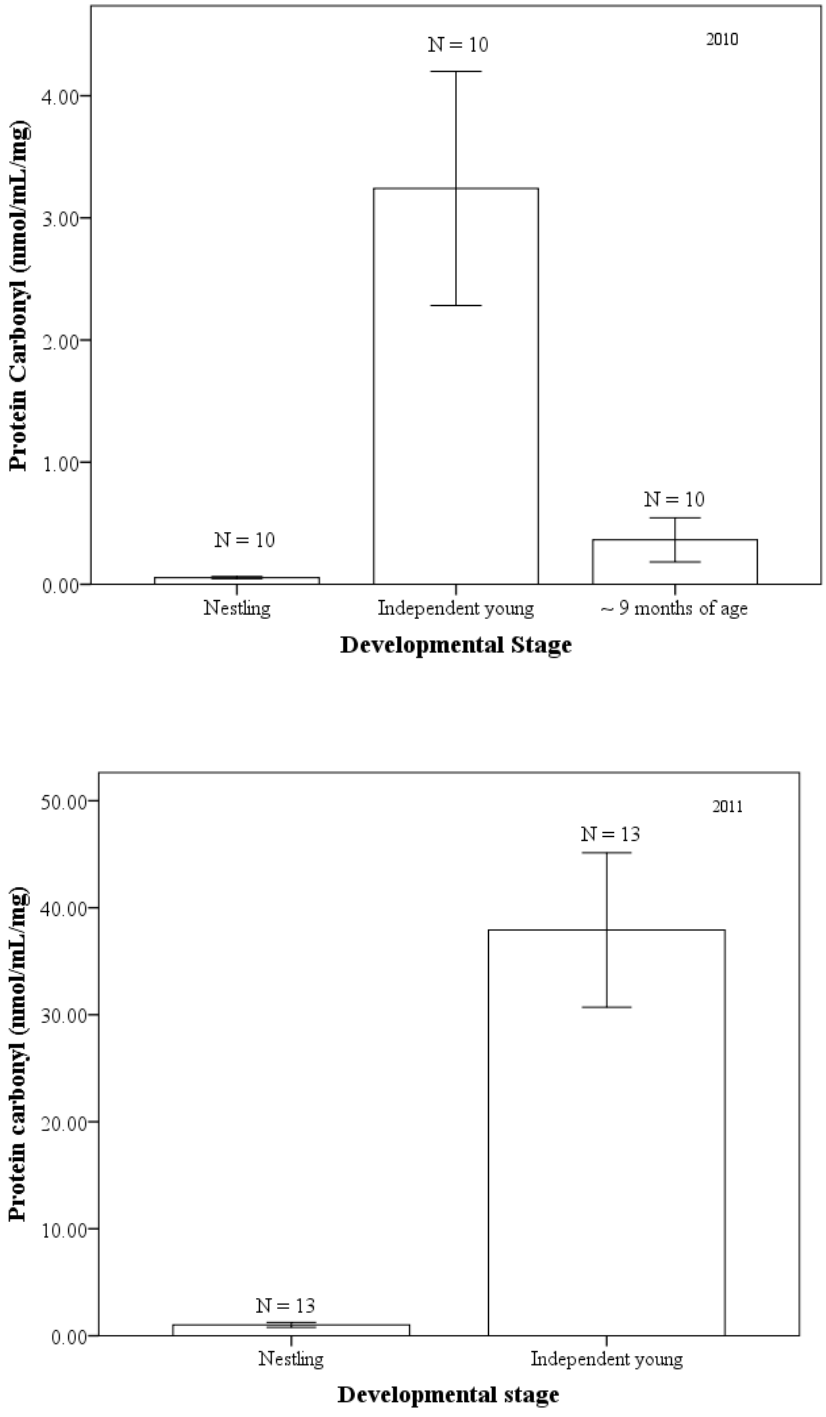


Figure 2: Absolute protein carbonyl levels during early developmental stages varied markedly between years. Panel A shows the means and standard errors of protein carbonyl levels in nestling, independent young, and 9 month old scrub-jays in 2010. Panel B shows the means and standard errors of protein carbonyl levels in nestling and independent young scrub-jays in 2011. Note difference in scale.

Post-hoc Tukey tests of the 2010 data revealed that protein carbonyl levels of nestlings (N = 65, 0.04 ± 0.003 nmol/mL/mg) were significantly lower than nutritionally independent young (N = 34, 2.36 ± 0.53 nmol/mL/mg; $P < 0.0001$) which were significantly higher than carbonyl levels of 9 month old birds (N=27, 0.86 ± 0.31 nmol/mL/mg; $P = 0.007$). However, there was no statistical difference between nestling and 9 month old birds ($P = 0.07$).

Survival and oxidative state

Neither TAC nor protein carbonyl levels at any stage of development were predictive of survival to the subsequent developmental stage. Further, changes in protein carbonyl levels between developmental stages were not related to subsequent survival (Table 1).

Table 1: Oxidative state of Florida scrub-jays at early developmental stages was not predictive of survival in either 2010(a) or 2011(b).

a)

	Nestling to Nutritional Independence				Nutritional Independence to 9 months of age				Nutritional Independence to 12 months of age			
Oxidative Measures	**Survived**	**Died**	**χ^2**	**P**	**Survived**	**Died**	**χ^2**	**P**	**Survived**	**Died**	**χ^2**	**P**
Protein carbonyl	43	22	0.04	0.84	29	5	3.59	0.06	22	12	0.37	0.52
Change in carbonyl	17	1	2.43	0.12	11	1	0.87	0.35				

b)

	Nestling to Nutritional Independence				Nutritional Independence to 9 months of age				Nutritional Independence to 12 months of age			
Oxidative Measures	**Survived**	**Died**	**χ^2**	**P**	**Survived**	**Died**	**χ^2**	**P**	**Survived**	**Died**	**χ^2**	**P**
TAC	10	34	0.06	0.80								
Protein carbonyl	27	13	0.04	0.84	2	17	1.70	0.19	3	16	2.30	0.13
Change in carbonyl	1	7	0.68	0.41	-	-	-	-				

DISCUSSION

In our consideration of adult jays, we found that oxidative damage to proteins was negatively correlated with age, whereas TAC was positively correlated with age. This corresponds with Wilcoxen et al. (2010b) who found that the population of scrub-jays used in this study, appeared to be culled of individuals with weaker immunocompetence, as older birds were characterized by almost uniformly high bacteria killing ability, whereas younger birds exhibited marked variance in this ability. However, our analysis of survival shows no indication that individuals with relatively high oxidative damage are being eliminated from the population. Instead, we found that oxidative damage levels of individuals change significantly between developmental stages, and we suggest that the ability to both reduce (via replacement of damaged proteins) and maintain low levels of oxidative damage may be responsible for the pattern of low oxidative damage associated with older birds in this population. Alternatively, without data on carbonyl levels in adult birds during these time periods (June-Jan), it is impossible to rule out that the patterns of oxidative damage in our data are reflective of a seasonal stress. Further work in adult birds during these summer and fall months is necessary to eliminate this possibility.

Elevated levels of plasma antioxidants in older birds were reported in a similar study of a cross-section of captive red-legged partridges (*Alectoris rufa;* Alonso-Alvarez et al. 2010). The authors suggested that their results reflected a compensatory mechanism for the “challenges of aging.” An increase in plasma TAC as the result of an inflammatory or stress response has been well documented in other bird species (Lin et al. 2004a,b, Hõrak et al. 2007, Maurice

et al. 2007). Given these findings, we suggest that the increased ability to resist an oxidative attack in older scrub-jays may be a response to the "challenge of aging." Specifically, this may reflect a mechanism to mitigate the age-related increases in ROS that have been characterized in most major organ systems across taxa (Gomi et al. 1993, Bejma and Ji 1999, Bejma et al. 2000, Driver et al. 2000, Zhang et al. 2003), rather than an indication of higher quality individuals.

To our knowledge, ours is the first study to show a significant change in oxidative damage levels across early developmental stages in a free living species of bird. Of importance is both the dramatic increase of protein carbonyls between the nestling and nutritional independence stages, as well as their subsequent decrease when measured again at 9 months of age. Under stressful conditions, ROS production and resultant damages increase (Herbert 1994, Heise et al. 2003, Costantini and Møller 2009, Costantini et al. 2011). During the life stage characterized by the highest levels of oxidative damage, the young jays are in the process of becoming self-sufficient and learning to forage and survive independent of their parents; a time that is undoubtedly challenging. Thus, perhaps it is not surprising that oxidative damage levels of these birds are elevated. However, the significant decrease in protein carbonyl levels at 9 months of age suggests an improved competency in self-maintenance. The fact that we did not detect any survival differences in birds that differed in their abilities to reduce their oxidative damage levels suggests an alternative interpretation of our results (see below).

While increased levels of oxidative damage to tissues have been positively associated with age across a wide range of vertebrates (e.g., Hamilton et al. 2001, Gianni et al. 2004, Valls 2005), it is possible that these age-related damages are not reflected in plasma, as damage has been shown to vary widely among tissue types (Sohal et al. 1995, Gaál et al. 1996, Costantini 2008). Given the high turnover rate of constituents within plasma, the plasma oxidative damage levels may reflect instantaneous condition rather than a cumulative measure, such as might be measured in tissues that experience relatively slower turnover (Mayo et al. 2003, Nussey et al. 2009). This interpretation was applied to results of a human-based study whose findings paralleled our own, as older subjects had lower levels of oxidative damage to proteins in plasma, and higher concentrations of plasma antioxidants (Garibaldi et al. 2001). This alternative interpretation may further explain why we failed to find increased oxidative damage to DNA with age given that the assay used assesses a marker of damage to DNA within blood plasma.

Finally, the between-year difference in oxidative damage was surprising. Birds in 2010 had markedly lower levels of protein carbonyls at both the nestling and nutritional independence stages, relative to birds in 2011. This is particularly interesting when considered in light of the pronounced differences in environmental conditions between these years. Whereas 2010 was an excellent year in terms of nestling production and survival of fledglings, 2011 was a considerably poorer year (see Schoech 2009 for discussion of good versus bad years in this population). The masses of 2010 nestlings at day 11 were

significantly greater than nestlings in six of the previous nine years (2001-2009) and the average nestling tarsus length was greater than that of nestlings in all of the previous seven years (2003-2009), suggesting that environmental conditions, including resource availability, were favorable during the breeding season in 2010. In comparison, 2011 nestling mass was lower and tarsi were smaller or equal to 9 of the 11 previous years.

It will be of interest to determine whether adult birds (older than 2) hatched in either 2010 or 2011 show similar patterns of oxidative health state over the years or if lifelong patterns and coping strategies for both production and alleviation of oxidative damage were established by their very different developmental conditions. Experimental manipulation of oxidative damage levels of individuals during early developmental stages might reveal whether differences in oxidative state (such as those noted above) have long-term impacts on individuals (e.g., body condition, survival, and the ability to combat ROS).

ACKNOWLEDGEMENTS

We thank all at Archbold Biological Station, especially R. Bowman and H. Swain. Special thanks to J. A. Cole, M. A. Rensel, B. C. Jones, S. E. Bebus, E. K. Elderbrock, E. S. Bridge and R. Hanauer. The research herein was funded by a National Science Foundation collaborative grant to S. J. Schoech and E. S. Bridge [IOS-0919899 and -0919528], as well as a National Science Foundation Doctoral Dissertation Improvement Grant to R. S. Heiss and S. J. Schoech [IOS-1110315] and a Sigma Xi grant to R. S. Heiss [G20111015157508].

REFERENCES

Alonso-Alvarez, C., L. Pérez-Rodríguez, J. T. García, J. Viñuela, and R. Mateo. 2010. Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. *Physiological and Biochemical Zoology* 83:110-118.

Beckman, K. B., and B. N. Ames. 1998. The free radical theory of aging matures. *Physiological Reviews* 78:547-581.

Bejma, J., and L. L. Ji. 1999. Aging and acute exercise enhance free radical generation in rat skeletal muscle. *Journal of Applied Physiology* 87:465–470.

Bejma, J., P. Ramires, and L. L. Ji. 2000. Free radical generation and oxidative stress with ageing and exercise: differential effects in the myocardium and liver. *Acta Physiologica Scandinavica* 169:343–351.

Berlett, B. S., and E. R. Stadtman. 1997. Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry* 272:20313–20316.

Costa, V., A. Quintanilha, and P. Moradas-Ferreira. 2007. Protein oxidation, repair mechanisms and proteolysis in *Saccharomyces cerevisiae. IUBMB Life*. 59:293–298.

Costantini, D. 2008. Oxidative stress in ecology and evolution: Lessons from avian studies. *Ecology Letters.* 11:1238–1251.

Costantini, D. 2010. Effects of diet quality on serum oxidative status and body mass in male and female pigeons during reproduction. *Comparative Biochemistry and Physiology, A.* 156:294-299.

Costantini, D., S. Casagrande, S. De Filippis, G. Brambilla, A. Fanfani, J. Tagliavini, and G. Dell'Omo. 2006. Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *Journal of Comparative Physiology, B.* 176:329-337.

Costantini, D., and G. Dell'Omo. 2006a. Effects of T-cell-mediated immune response on avian oxidative stress. *Comparative Biochemistry and Physiology, A.* 145:137-142.

Costantini, D. and G. Dell'Omo. 2006b. Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *Journal of Comparative Physiology, B.* 176:575-579.

Costantini, D., V. Marasco, and A. P. Møller. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. *Journal of Comparative Physiology, B.* 181:447–456.

Costantini, D., and A. P. Møller. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. *Comparative Biochemistry and Physiology, A.* 153:339–344.

Devevey, G., N. Bruyndonckx, F. Houwald, A. Studer-Thiersch, and P. Christe. 2009. Age-specific variation of resistance to oxidative stress in the greater flamingo (*Phoenicopterus ruber roseus*). *Journal of Ornithology* 15:251–254.

Driver, A. S., P. R. S. Kodavanti, and W. R. Mundy. 2000. Age-related changes in reactive oxygen species production in rat brain homogenates. *Neurotoxicology and Teratology* 22:175–181.

Forster, M. J., A. Dubey, K. M. Dawson, W. A. Stutts, H. Lal, and R. S. Sohal. 1996. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. *Proceedings of the National Academy of Sciences, USA* 93:4765–4769.

Friguet, B. 2002. Protein repair and degradation during aging. *Scientific World Journal* 2:248–254.

Friguet, B. 2006. Oxidized protein degradation and repair in ageing and oxidative stress. *FEBS Letters* 580:2910–2916.

Friguet, B., A. L. Bulteau, N. Chondrogianni, M. Conconi, and I. Petropoulos. 2000. Protein degradation by the proteasome and its implications in aging. *Annuals of the New York Academy of Sciences* 908:143–154.

Gaál, T., B. K. Speake, M. Mezes, R. C. Noble, P. F. Surai, and P. Vajdovich. 1996. Antioxidant parameters and ageing in some animal species. *Comparative Haematology International* 6:208-213.

Garibaldi, S., S. Valentini, I. Aragno, M. A. Pronzato, N. Traverso, and P. Odetti. 2001. Plasma protein oxidation and antioxidant defense during aging. *International Journal for Vitamin and Nutrition Research* 71:332-338.

Gianni, P., K. J. Jan, M. J. Douglas, P. M. Stuart, and M. A. Tarnopolsky. 2004. Oxidative stress and the mitochondrial theory of aging in human skeletal muscle. *Experimental Gerontology* 39:1391–1400.

Gomi, F., H. Utsumi, A. Hamada, and M. Matsuo. 1993. Aging retards spin clearance from mouse brain and food restriction prevents its age-dependent retardation. *Life Sciences* 52:2027–2033.

Gredilla, R., V. A. Bohr, and T. Stevnsener. 2010. Mitochondrial DNA repair and association with aging - an update. *Experimental Gerontology* 45:478-488.

Hamilton, M. L., H. Van Remmen, J. A. Drake, H. Yang, Z. M. Guo, K. Kewitt, C. A. Walter, and A. Richardson. Does oxidative damage to DNA increase with age? *Proceedings of the National Academy of Sciences, USA* 98:10469–10474.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology* 11:298-300.

Heise, K., S. Puntarulo, H. Pörtner, and D. Abele. 2003. Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. *Comparative Biochemistry and Physiology, C* 134:79–90.

Herbert, V. 1994. The antioxidant supplement myth. *American Journal of Clinical Nutrition* 60:157–158.

Herbert, V., S. Shaw, and E. Jayatilleke. 1994. Vitamin C supplements are harmful to lethal for the over 10% of Americans with high iron stores. *FASEB Journal* 8:A678.

Hõrak, P., L. Saks, M. Zilmer, U. Karu, and K. Zilmer. 2007. Do dietary carotenoids alleviate the cost of immune activation? An experiment with greenfinches. *American Naturalist* 170:625-635.

Kregel, K. C., and H. J. Zhang. 2007. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* 292:R18-R36.

Lin H., E. Decuypere, and J. Buyse. 2004a. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*). 1. Chronic exposure. *Comparative Biochemical Physiology, B.* 139:737–744.

Lin H., E. Decuypere, and J. Buyse. 2004b. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*). 2. Short-term effect. *Comparative Biochemical Physiology, B.* 139:745–751.

Markó, G., D. Costantini, G. Michl, and J. Török. 2011. Oxidative damage and plasma antioxidant capacity in relation to body size, age, male sexual traits and female reproductive performance in the collared flycatcher (*Ficedula albicollis*). *Journal of Comparative Physiology, B.* 81:73–81.

Maurice, D., S. F. Lightsey, J. E. Toler, and S. Canty. 2007. Effect of chronic oxidative/corticosterone-induced stress on ascorbic acid metabolism and total antioxidant capacity in chickens (*Gallus gallus domesticus*). *Journal of Animal Physiology and Animal Nutrition* 91:355–360.

Mayo, J., D. Tan, R. M. Sainz, M. Natarajan, S. Lopez-Burillo, and R. J. Rieter. 2003. Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants. *Biochimica et Biophysica Acta* 1620:139–150.

McDonald, D. B., J. W. Fitzpatrick, and G. E. Woolfenden. 1996. Actuarial senescence and demographic heterogeneity in the Florida scrub jay. *Ecology* 77:2373-2381.

Monaghan, P., N. B. Metcalfe, and R. Torres. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters* 12:75-92.

Newton, R. K., J. M. Ducore, and R. S. Sohal. 1989a. Effect of age on endogenous DNA single-strand breakage, strand break induction and repair in the adult housefly, *Musca domestica*. *Mutation Research* 219:113–120.

Newton, R. K., J. M. Ducore, and R. S. Sohal. 1989b. Relationship between life expectancy and endogenous DNA single-strand breakage, strand break induction and DNA repair capacity in the adult housefly, *Musca domestica*. *Mechanisms of Ageing and Development* 49:259–270.

Nussey, D. H., J. M. Pemberton, J. G. Pilkington, and J. D. Blount. 2009. Life history correlates of oxidative damage in a free-living mammal population. *Functional Ecology* 23:809-817.

Park, S. H., H. J. Kang, H. S. Kim, M. J. Kim, J. I. Heo, J. H. Kim, Y. J. Kho, S. Kim, J. Kim, J. B. Park, and J. Y. Lee. 2011. Higher DNA repair activity is related with longer replicative life span in mammalian embryonic fibroblast cells. *Biogerontology* 12:565–579.

Petropoulos, I., and B. Friguet. 2005. Protein maintenance in aging and replicative senescence: a role for the peptide methionine sulfoxide reductases. *Biochimica et Biophysica Acta* 1703:261 – 266.

Resnick, A. Z. and L. Packer. 1994. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods in Enzymology* 233:357-363.

Saino, N., M. Caprioli, M. Romano, G. Boncoraglio, D. Rubolini, R. Ambrosini, A. Bonisoli-Alquati, and A. Romano. 2011. Antioxidant defenses predict long-term survival in a passerine bird. *PloS One* 6:1-7.

Schoech, S. J. 1996. The effect of supplemental food on body condition and the timing of reproduction in a cooperative breeder, the Florida Scrub-Jay. *Condor* 98:234-244.

Schoech, S. J. 2009. Food supplementation experiments: A tool to reveal mechanisms that mediate timing of reproduction. *Integrative and Comparative Biology* 49:480-492.

Schoech, S. J., and R. Bowman. 2003. Does differential access to protein influence differences in timing of breeding of Florida Scrub-Jays (*Aphelocoma coerulescens*) in suburban and wildland habitats? *Auk* 120:1114-1127.

Shen, J., P. Deininger, J. D. Hunt, and H. Zhao. 2007. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) as a potential survival biomarker in patients with nonsmall-cell lung cancer. *Cancer* 109:574–580.

Sohal, R. S., R. J. Mockett, and W. C. Orr. 2002. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. *Free Radical Biology and Medicine* 33:575–586.

Sohal, R. S., S. Agarwal, A. Dubey, and W. C. Orr. 1993. Protein oxidative damage is associated with life expectancy of houseflies. *Proceedings of the National Academy of Sciences, USA* 90:7255–7259.

Sohal, R. S., S. Agarwal, and B. H. Sohal. 1995. Oxidative stress and aging in the Mongolian gerbil (*Meriones unguiculatus*). *Mechanisms of Aging and Development.* 81:15-25.

Stadtman, E. R. 2001. Protein oxidation in aging and age-related diseases. *Annuals of the New York Academy of Sciences* 928:22–38.

Stadtman, E. R., and R. L. Levine. 2000. Protein oxidation. *Annuals of the New York Academy of Sciences* 899:191–208.
Starke-Reed, P. E., and C. N. Oliver. 1989. Protein oxidation and proteolysis during aging and oxidative stress. *Achieves of Biochemistry and Biophysics* 275:559–567.

Takabayashi, F., S. Tahara, T. Kaneko, Y. Miyoshi, and N. Harada. 2004. Accumulation of 8-oxo-2'-deoxyguanosine (as a biomarker of oxidative DNA damage) in the tissues of aged hamsters and change in antioxidant enzyme activities after single administration of N-nitrosobis(2-oxopropyl) amine. *Gerontology* 50:57–63.

Valls, V., C. Peiro, P. Muniz, and G. T. Saez. 2005. Age-related changes in antioxidant status and oxidative damage to lipids and DNA in mitochondria of rat liver. *Process Biochemistry* 40:903–908.

Whitehead, I., and T. A. Grigliatti. 1993. A correlation between DNA repair capacity and longevity in adult *Drosophila melanogaster*. *Journal of Gerontology* 48:B124–B132.

Wilcoxen, T. E., R. K. Boughton, and S. J. Schoech. 2010a. Older can be better: physiological costs of paternal investment in the Florida scrub-jay. *Behavioral and Ecology and Sociobiology* 64:1527-1535.

Wilcoxen, T. E., R. K. Boughton, and S. J. Schoech. 2010b. Selection on innate immunity and body condition in Florida scrub-jays throughout an epidemic. *Biology Letters* 6:552-554.

Woolfenden, G. E. and J. W. Fitzpatrick. 1996. Florida scrub-Jay (*Aphelocoma coerulescens*), The Birds of North America Online (Poole, A. and F. Gill, eds.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online.

Yan, L. J. and R. S. Sohal. 1998. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proceedings of the National Academy of Sciences, USA* 95:12896–12901.

Yasuda, K., H. Adachi, Y. Fujiwara, and N. Ishii. 1999. Protein carbonyl accumulation in aging dauer formation-defective (*daf*) mutants of *Caenorhabditis elegans*. *Journal of Gerontology, A.* 54:B47– B51..

Zhang, H. J., L. Xu, V. J. Drake, L. Xie, L. W. Oberley, and K. C. Kregel. 2003. Heat-induced liver injury in old rats is associated with exaggerated oxidative stress and altered transcription factor activation. *FASEB Journal* 17:2293–2295.

CHAPTER 6: CONCLUSIONS

The results of the studies presented in this dissertation characterize relationships between oxidative status and several key components of life history stages in Florida Scrub-jays. This work represents a significant contribution to the field as many of the measures were assessed for the first time in free-living birds.

The goal of the first studies, presented in chapters 2 and 3, was to assess the relationship between reproductive effort and oxidative status. In chapter 2, I discussed the ways in which oxidative damage could be both predictive of, and result from, reproductive effort. I found both of these predictions to be supported by my data but only in males. In chapter 3, I followed up on these findings and attempted to attenuate the oxidative cost of reproduction by supplementing a subset of breeder males with antioxidants, specifically carotenoids. Additionally, I expanded my assessment of oxidative status to include three measures of oxidative state (oxidative damage to protein and DNA, as well as total plasma antioxidant capacity). Birds supplemented with carotenoids did not have lower oxidative damage levels post-breeding relative to their non-supplemented counterparts. However, I did uncover a relationship between change in oxidative damage levels from pre- to post-breeding with reproductive effort and treatment, such that supplemented birds had lower overall levels of oxidative damage (combined DNA and protein) than nonsupplemented birds. Unfortunately, these findings must be interpreted with caution as sample sizes for this analysis were small. Arguably one of the most important findings presented in this chapter was the lack of correlations among measures of oxidative status. This stresses the

importance of assessing multiple measures of oxidative status and undermines assumptions made based upon a single measure.

In chapter 4 I move to another important life history stage to assess the impacts of oxidative status on development. In this study I supplemented a subset of nestlings with carotenoids to assess whether these antioxidants would affect the growth and oxidative balance relationship in a developing passerine. I also further pursued multiple measures of oxidative status to determine whether any were correlated. I found no effect of treatment upon growth or oxidative status. Further, similar to findings from adult birds, no measures of oxidative status were correlated. Thus, my data suggest that carotenoids are of little importance to growth and the maintenance of oxidative stress in developing scrub-jay nestlings.

Finally, in chapter 5 I assessed whether oxidative status varied with age across individuals. The accumulation of oxidative damage across time has been associated with numerous disease states, as well as aging. While working on my previous chapters I observed a wide range of variation in oxidative status among individuals across age classes; particularly among individuals at early developmental stages. In this chapter I explored this variation using both a cross-sectional approach to include known age individuals across the adult spectrum of ages, and a repeated measures design to explore how variation of oxidative status at early developmental stages might affect survivorship. Interestingly, my data do not agree with the free-radical theory of aging as oxidative damage to proteins was negatively correlated with age, oxidative damage to DNA was

independent of age, and total antioxidant capacity was positively correlated with age. I also found that jays suffered a significant increase in oxidized proteins as they transitioned from nestling to a nutritionally independent stage. However, these levels of oxidative damage had returned to near nestling level again half a year later, suggesting that the replacement of oxidized proteins is an essential function during this time period. I found no relationship between survival and oxidative status during the early developmental period which suggests that birds experiencing high levels of oxidative stress are not being eliminated from the population as the above noted age-based correlations appear to suggest.

The integral combination of laboratory and fieldwork required for this dissertation work has allowed me to address a large gap of knowledge in this field and to assess the implications of oxidative damage in a free-living bird. The results of this dissertation provide a strong framework for the continued monitoring of long-term effects of oxidative status in a free-living population of birds.