SPERM TELOMERE DYNAMICS: NATURAL VARIATION AND SENSITIVITY TO ENVIRONMENTAL INFLUENCES IN HOUSE SPARROWS (*PASSER DOMESTICUS*)

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North Dakota State University's regulations and meets the accepted

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Understanding the mechanisms that contribute to variation in lifespan is of central importance to diverse fields including life history theory. Although the causes of aging are not fully understood, telomere dynamics (length and loss rate) is a potentially critical mechanism underlying longevity. Telomeres are highly conserved, non-coding regions of DNA at the ends of eukaryotic chromosomes. Telomere loss occurs throughout life due to accumulating oxidative damage and normal cell replication. When telomeres reach a critically short length, they stop dividing and functioning normally.

While early life telomere length is predictive of lifespan in birds, the mechanism of inheritance of telomere length is unknown. One hypothesized mechanism is by direct transfer from gamete telomeres. However, very little is known about telomere dynamics in gametes.

Stress exposure has been shown to accelerate telomere loss and reduce longevity, particularly when stress is experienced early in life. Exposure to elevated glucocorticoid hormones during activation of the stress response is thought to lead to increased oxidative damage, and thereby accelerate telomere loss. Sperm are particularly sensitive to oxidative damage. Therefore, exposure to stress may accelerate aging within individuals, but also may accelerate sperm telomere loss and thereby impact the telomere dynamics of their offspring.

To test this hypothesis, I measured natural variation in sperm telomere length and offspring early life telomeres, sperm telomere length in response to acute and chronic stress exposure, and the relationship between stress sensitivity and telomere length. In free-living sparrows, I found no relationship between paternal sperm telomere length and offspring early life telomere dynamics. Across studies, there was a consistent positive correlation between blood and sperm telomere length, suggesting that sperm telomeres may decline with age in birds. I also

found variation in sperm telomere length across the breeding season, and no relationship between stress sensitivity and sperm telomere length. Finally, I found that while sperm telomere length in free-living birds exceeded blood telomere length, in captivity sperm telomeres were equal length or shorter than blood telomeres, potentially related to the duration of captivity. These findings suggest that sperm telomeres are sensitive to environmental factors including stress exposure.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Telomere Basics

The study of telomeres began in the early 1930's when Barbara McClintock and Hermann Muller noticed that broken chromosome ends could fuse with other broken ends. They also realized that natural chromosome ends could never fuse with broken or other natural ends (McClintock 1931; Blackburn 2006). Thus, the protective function of telomeres in chromosome capping became evident even before the structure of DNA was known. Each chromosome end is equipped with a telomere, a highly conserved, repetitive non-coding sequence that binds to shelterin proteins and forms a t-loop; this allows it to be easily distinguished as a true chromosome end (Haussmann and Marchetto 2010). Without a telomere, the natural end of a chromosome might be recognized as a double-stranded DNA break in need of repair (Blackburn 2005; Haussmann and Marchetto 2010).

The next several decades brought more groundbreaking discoveries about telomeres and telomere dynamics, eventually leading to the 2009 Novel Prize in Physiology or Medicine being awarded to telomere researchers Elizabeth Blackburn, Carol Greider, and Jack Szostak for their work on chromosome protection by telomeres and telomerase. Telomeres protect DNA during replication but are subject to incomplete terminal replication themselves. During a replication event, a piece of the lagging strand is not replicated and remains longer than the newly synthesized strand. This piece is called the G-strand overhang and is important in formation of the t-loop to complete the protective cap (Haussmann and Marchetto 2010). It also means that telomeres progressively shorten with repeated replication events. In fact, human telomeres shorten by 50-300 nucleotides per replication, but of these lost nucleotides only about 10 are forfeited to incomplete terminal replication (Haussmann and Marchetto 2010). The majority of

telomere loss is likely caused by oxidative damage, as telomeres are guanine-rich and particularly vulnerable to oxidative damage (Haussmann and Marchetto 2010). Regardless of the relative contribution of each mechanism, telomere loss is consistently associated with aging and senescence (Monaghan 2010).

Telomere loss occurs throughout an individual's life in many taxa (including birds, reptiles, fish, and mammals), and loss rate is consistently highest during development, when cells are dividing most rapidly (Monaghan 2010). However, individuals of the same age can differ markedly in telomere length. In zebra finches, early life telomere length is predictive of lifespan; young finches with the longest telomeres live the longest (Heidinger et al. 2012). This means that telomere length and loss rate (collectively termed telomere dynamics) can be useful tools for assessing differences between individuals or populations even though absolute telomere length is not a reliable indicator of an organism's chronological age.

Genome instability eventually results from telomere loss, when one or more of a cell's telomeres shorten to a critical length. This leads to loss of cell function, either by replicative senescence or by apoptosis (Monaghan and Haussmann 2006). Replicative senescence is a state in which a cell no longer divides and often has altered functionality (Monaghan 2010). This state is triggered when telomeres are too short to form t-loops and thus chromosomes become uncapped (Bekaert et al. 2005; Monaghan 2010).

Telomere loss is usually considered a one-way street, but restoration can occur in some cases. There are several known or probable mechanisms for telomere restoration, the most common of which depends on the enzyme telomerase (Monaghan 2010). Telomerase expression is mostly downregulated after early development but remains active in dividing tissues, including stem cells and germ line cells (Monaghan and Haussmann 2006; Haussmann et al. 2007). If

telomerase can restore telomere length and prevent chromosome uncapping, it would seem advantageous to maintain it in all tissues. In reality, telomerase is sometimes activated in adult somatic cells whose chromosomes have become uncapped – these cells become malignant rather than senescent (Monaghan and Haussmann 2006). Telomere shortening and replicative senescence therefore play a critical role in preventing complications that could arise from DNA damage and chromosome instability. In addition to chromosome capping, telomeres act as chromosome wardens; the damage they accumulate is indicative of the overall level of DNA damage from various sources. Cell replication and transcription are usually halted before the damage becomes destructive or unmanageable (Haussmann and Marchetto 2010).

Telomere shortening is a firmly established cause of cellular senescence *in vitro*, but telomere dynamics (length and loss rate) are likely a critical mechanism underlying organismal aging as well. Recent avian studies are helping to solidify the link between telomere loss and organismal aging. For example, Asghar et al. (2015) found that great reed warblers with chronic avian malaria had reduced lifespan and shorter telomeres as compared to uninfected individuals. Other birds also suffer reduced longevity from exposure to stressful circumstances, especially when the stress is experienced early in life (Monaghan et al. 2012). This may be mediated by telomere loss, as a similar exposure to stress during the juvenile stage accelerated telomere loss in a long-lived seabird (Herborn et al. 2014).

The role of telomere dynamics in organismal aging, and the factors that mediate, accelerate, and reduce telomere loss are all exciting areas of study with many yet unexplored opportunities. My research focuses on an emerging area in telomere research with roots in a familiar idea. Here I describe my approach to begin answering a large question: does stress experienced by parents influence rate of aging in their offspring?

House Sparrows (*Passer domesticus*)

House sparrows are medium-sized seed-eating passerines in the genus *Passer*, Old World sparrows, with a native range spanning Europe, Asia, and North Africa (Anderson 2006). Now the most widespread land bird in the world, house sparrows have lived in close association with humans for many thousands of years (Anderson 2006). House sparrows are nonmigratory, semi-colonial, secondary cavity nesters; they often nest in buildings of all kinds, and populations can be very dense in areas with plentiful food, such as agricultural and urban areas (Anderson 2006). They were introduced to North America from Europe repeatedly across the latter half of the 19th century, and populations were widespread across the United States and Canada by 1915 (Anderson 2006). While originally thought to be helpful in controlling crop pests, house sparrows are only insectivorous during chick rearing and are themselves crop pests for most of the year (Anderson 2006). Now widely considered nuisance birds, house sparrows are most populous across the Midwest, where agriculture is particularly prevalent; populations in the upper Midwest are also among the largest in North America (Anderson 2006).

Due to their relative abundance and now-global distribution, house sparrows have served as subjects for a wide range of studies, including transcriptome sequencing, invasion biology, and stress responses (e.g. Lendvai et al. 2007; Ekblom et al. 2014; Liebl et al. 2015). To address questions about longevity, telomere dynamics, and reproduction in birds, free-living house sparrows seemed an ideal non-model subject. House sparrows have a modal lifespan of 2 years and maximum of over 10, allowing us to follow known-age individuals across their entire lives with good variation in individual longevity (Anderson 2006). They are accustomed to human presence, and have multiple clutches across the summer, with chicks fledging 12-15 days after hatching (Anderson 2006). In 2014, I helped establish the Heidinger Lab nest box population on

North Dakota State University agricultural sites in Fargo, ND. In our population, nest boxes are consistently occupied, chicks are easily accessible for repeated sampling and banding, and adults can be captured on the nest using my custom nest box trap (see Chapter 3). Our median brood size is 4 chicks, and we typically band 200 or more chicks per year in addition to fledglings and adults (pers. obs.). All of my studies were conducted in either free-living house sparrows at our field sites, or house sparrows taken into captivity from other sites in Fargo.

Figure 1. An adult male house sparrow guarding his nest in a saguaro cactus near Tucson, AZ.

Specific Questions

1. How are sperm and blood telomere length related, and how repeatable is sperm telomere length, within individuals?
2. How is sperm telomere length related to stress sensitivity? How is stress response influenced by stressor type?
3. Does stress experience impact sperm telomere dynamics? Are these effects dependent on duration of stressor exposure?
4. Is offspring early life telomere length related to paternal sperm telomere length?

Telomere length is most commonly measured in blood (leukocytes in mammals, erythrocytes in birds) as it is an easy tissue to sample noninvasively and has high cellular turnover (Haussmann et al. 2007). In adult animals, telomere loss rate is expected to be higher in highly proliferative tissues such as blood, gonads, and intestines, as compared to tissues with low cell division such as skeletal muscle and liver (Haussmann et al. 2007; Schmidt et al. 2016). Few studies have compared telomere length between tissues in birds, and in house sparrows it is unknown whether telomere length is correlated between tissues (Schmidt et al. 2016).

Sperm telomere length is of particular interest as direct inheritance of germ line telomeres is one hypothesized pathway of telomere inheritance (Haussmann and Heidinger 2015). Gonadal tissue is highly proliferative and also expresses telomerase throughout life in short-lived birds (Haussmann et al. 2007). Sperm are highly susceptible to oxidative damage, but upregulated telomerase expression in the gonad may protect gamete telomeres against damage caused by increased oxidative stress (Almbro et al. 2011; Losdat et al. 2011; Haussmann and Heidinger 2015).

Human studies suggest that sperm telomere length increases with age, while leukocyte telomere length decreases, and that older fathers produce offspring with longer telomeres (Kimura et al. 2008; Aston et al. 2012; Eisenberg et al. 2012; Ferlin et al. 2013; Yang et al. 2015). Beyond this, little is known about potential differences in telomere dynamics between tissues, partly due to the difficulty of obtaining repeat samples of many tissues (Reichert et al. 2013; Schmidt et al. 2016). To date, sperm telomere length has only been reported in humans, pigs, and cattle (Kozik et al. 1998; Ferlin et al. 2013).

Telomere loss occurs throughout life due to accumulating oxidative damage and normal DNA replication processes (Monaghan 2014; Zhao et al. 2014; Haussmann and Heidinger 2015). Stress exposure has been shown to accelerate telomere loss and reduce longevity, particularly when stress is experienced in early life (Epel et al. 2004; Haussmann and Marchetto 2010; Tyrka et al. 2010; Herborn et al. 2014; Asghar et al. 2015). The impact of stress experience on telomere dynamics during adulthood is less studied, but there is evidence that stress can accelerate telomere loss in adult birds (Monaghan et al. 2012; Hau et al. 2015). However, mild stress can also increase longevity (Marasco et al. 2015). These hormetic effects may be the result of increased telomerase expression in response to stress (Monaghan 2014; Haussmann and Heidinger 2015). Elevation of circulating glucocorticoid hormones in response to stress, including the primary avian stress hormone corticosterone (CORT), leads to increased oxidative damage (Costantini et al. 2011; Monaghan 2014). Therefore, individual differences in telomere loss rate may be partially mediated by differences in sensitivity to stress.

In a typical avian acute stress response, elevation of CORT begins within minutes of stressor exposure, and negative feedback at the hypothalamus and pituitary slowly reduce circulating glucocorticoids to baseline after reaching maximal levels within an hour (Romero and

Romero 2002). Dysregulation of the HPA axis can occur when stressors are frequent or prolonged; when this occurs, the animal is said to be experiencing chronic stress (Rich and Romero 2005; Miller et al. 2007). This dysregulation of the HPA axis can manifest as either depressed or elevated CORT (baseline, stress-induced, and negative feedback), and may depend on stressor type and timing in relation to stressor onset (Breuner et al. 1999; Clinchy et al. 2004; Rich and Romero 2005; Miller et al. 2007; Dickens et al. 2009). While context-dependent stress responsiveness is a topic of broad interest, few studies have considered how individual stress responsiveness varies with stressor type in birds (Silverin 1998; Cockrem and Silverin 2002a; Butler et al. 2009; Canoine et al. 2013).

In the following chapters, I address these questions in captive and free-living house sparrows. While intriguing questions remain as to what degree parental effects influence offspring aging, the mechanisms of inheritance of telomere length, and the sensitivity of sperm telomeres to environmental influences, I provide preliminary information in an emerging area of telomere biology.

Aging kills us all

Can dad's wisdom age his kids?

Sperm may be the key

References

Almbro M, Dowling DK, Simmons LW. 2011. Effects of vitamin E and beta-carotene on sperm competitiveness. Ecol. Lett. 14:891–895.

Anderson TR. 2006. Biology of the Ubiquitous House Sparrow: From Genes to Populations. Oxford University Press.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science 347:436–438.

Aston KI, Hunt SC, Susser E, Kimura M, Factor-litvak P, Carrell D, Aviv A. 2012. Divergence of sperm and leukocyte age-dependent telomere dynamics: Implications for male-driven evolution of telomere length in humans. Mol. Hum. Reprod. 18:517–522.

Bekaert S, De Meyer T, Van Oostveldt P. 2005. Telomere attrition as ageing biomarker. Anticancer Res. 25:3011–3022.

Blackburn EH. 2005. Telomeres and telomerase: Their mechanisms of action and the effects of altering their functions. FEBS Lett. 579:859–862.

Blackburn EH. 2006. A history of telomere biology. In: Telomeres. 2nd ed. Col Spring Harbor Monograph Archive. p. 45.

Breuner CW, Wingfield JC, Romero LM. 1999. Diel rhythms of basal and stress-induced cort in wild white-crowned sparrow. J. Exp. Zool. 284:334–342.

Butler LK, Bisson IA, Hayden TJ, Wikelski M, Romero LM. 2009. Adrenocortical responses to offspring-directed threats in two open-nesting birds. Gen. Comp. Endocrinol. 162:313–318.

Canoine V, Hayden TJ, Rowe K, Goymann W. 2013. The Stress Response of European Stonechats Depends on the Type of Stressor. Behaviour 139:1303–1311.

Clinchy M, Zanette L, Boonstra R, Wingfield JC, Smith JNM. 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proc. R. Soc. B Biol. Sci. 271:2473–2479.

Cockrem JF, Silverin B. 2002. Sight of a predator can stimulate a corticosterone response in the great tit (Parus major). Gen. Comp. Endocrinol. 125:248–255.

Costantini D, Marasco V, Møller AP. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181:447–456.

Dickens M, Romero LM, Cyr NE, Dunn IC, Meddle SL. 2009. Chronic stress alters glucocorticoid receptor and mineralocorticoid receptor mRNA expression in the European starling (Sturnus vulgaris) brain. J. Neuroendocrinol. 21:832–840.

Eisenberg DTA, Hayes MG, Kuzawa CW. 2012. Delayed paternal age of reproduction in humans is associated with longer telomeres across two generations of descendants. Proc. Natl. Acad. Sci. 109:10251–10256.

Ekblom R, Wennekes P, Horsburgh GJ, Burke T. 2014. Characterization of the house sparrow (Passer domesticus) transcriptome: a resource for mulecular ecology and immunogenetics. Mol. Ecol. Resour. 14:636–646.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. 101:17312–17315.

Ferlin A, Rampazzo E, Rocca MS, Keppel S, Frigo AC, De Rossi A, Foresta C. 2013. In young men sperm telomere length is related to sperm number and parental age. Hum. Reprod. 28:3370–3376.

Hau M, Haussmann MF, Greives TJ, Matlack C, Costantini D, Quetting M, Adelman JS, Miranda AC, Partecke J. 2015. Repeated stressors in adulthood increase the rate of biological ageing. Front. Zool. 12:1–10.

Haussmann MF, Heidinger BJ. 2015. Telomere dynamics may link stress exposure and ageing across generations. Biol. Lett. 11:20150396.

Haussmann MF, Marchetto NM. 2010. Telomeres: Linking stress and survival, ecology and evolution. Curr. Zool. 56:714–727.

Haussmann MF, Winkler DW, Huntington CE, Nisbet ICT, Vleck CM. 2007. Telomerase activity is maintained throughout the lifespan of long-lived birds. Exp. Gerontol. 42:610–618.

Heidinger BJ, Blount JD, Boner W, Griffiths K, Metcalfe NB, Monaghan P. 2012. Telomere length in early life predicts lifespan. Proc. Natl. Acad. Sci. 109:1743–1748.

Herborn KA, Heidinger BJ, Boner W, Noguera JC, Adam A, Daunt F, Monaghan P. 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. Proc. R. Soc. B Biol. Sci. 281:20133151.

Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, Cupples A, Hunkin JL, Gardner JP, Lu X, et al. 2008. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 4:1–9.

Kozik A, Bradbury EM, Zalensky A. 1998. Increased telomere size in sperm cells of mammals with long termin (TTAGGG)n arrays. Mol. Reprod. Dev. 51:98–104.

Lendvai AZ, Giraudeau M, Chastel O. 2007. Reproduction and modulation of the stress response: an experimental test in the house sparrow. Proc. R. Soc. B Biol. Sci. 274:391–397.

Liebl AL, Schrey AW, Andrew SC, Sheldon EL, Griffith SC. 2015. Invasion genetics: Lessons from a ubiquitous bird, the house sparrow passer domesticus. Curr. Zool. 61:465–476.

Losdat S, Richner H, Blount JD, Helfenstein F. 2011. Immune activation reduces sperm quality in the great tit. PLoS One 6.

Marasco V, Boner W, Heidinger B, Griffiths K, Monaghan P. 2015. Repeated exposure to stressful conditions can have beneficial effects on survival. Exp. Gerontol. 69:170–175.

McClintock B. 1931. Cytological observations of deficiencies involving known genes, translocations and an inversion in Zea mays. Mo. Agric. Exp. Res. Stn. Res. Bull. 163:4–30.

Miller GE, Chen E, Zhou ES. 2007. If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychol. Bull. 133:25–45.

Monaghan P. 2010. Telomeres and life histories: The long and the short of it. Ann. N. Y. Acad. Sci. 1206:130–142.

Monaghan P. 2014. Organismal stress, telomeres and life histories. J. Exp. Biol. 217:57–66.

Monaghan P, Haussmann MF. 2006. Do telomere dynamics link lifestyle and lifespan? Trends Ecol. Evol. 21:47–53.

Monaghan P, Heidinger BJ, D'Alba L, Evans NP, Spencer KA. 2012. For better or worse: reduced adult lifespan following early-life stress is transmitted to breeding partners. Proc. R. Soc. B Biol. Sci. 279:709–714.

Reichert S, Criscuolo F, Verinaud E, Zahn S, Massemin S. 2013. Telomere length correlations among somatic tissues in adult zebra finches. PLoS One 8:1–7.

Rich EL, Romero LM. 2005. Exposure to chronic stress downregulates corticosterone responses to acute stressors. AJP Regul. Integr. Comp. Physiol. 288:R1628–R1636.

Romero LM, Romero RC. 2002. Corticosterone Responses in Wild Birds : The Importance of Rapid Initial Sampling. Condor 104:129–135.

Schmidt JE, Sirman AE, Kittilson JD, Clark ME, Reed WL, Heidinger BJ. 2016. Telomere correlations during early life in a long-lived seabird. Exp. Gerontol. 85:28–32.

Silverin B. 1998. Behavioural and hormonal responses of the pied flycatcher to environmental stressors. Anim. Behav. 55:1411–1420.

Tyrka AR, Price LH, Kao H, Marsella SA, Carpenter LL. 2010. Childhood maltreatment and telomere shortening: Preliminary support for an effect of early stress on cellular aging. Biol. Psychiatry 67:531–534.

Yang Q, Zhao F, Dai S, Zhang N, Zhao W, Bai R, Sun Y. 2015. Sperm telomere length is positively associated with the quality of early embryonic development. Hum. Reprod. 30:1876–1881.

Zhao Z, Pan X, Liu L, Liu N. 2014. Telomere length maintenance, shortening, and lengthening. J. Cell. Physiol. 229:1323–1329.

CHAPTER 2: AVIAN SEMEN COLLECTION BY CLOACAL MASSAGE AND ISOLATION OF DNA FROM SPERM[1]

Abstract

Collection of semen may be useful for a wide range of applications including studies involving sperm quality, sperm telomere dynamics, and epigenetics. Birds are widely used subjects in biological research and are ideal for studies involving repeated sperm samples. However, few resources are currently available for those wishing to learn how to collect and extract DNA from avian sperm. Here we describe cloacal massage, a gentle, non-invasive manual technique for collecting avian sperm. Although this technique is established in the literature, it can be difficult to learn from the available descriptions. We also provide information for extracting DNA from avian semen using a commercial extraction kit with modifications. Cloacal massage can be easily used on any small- to medium-sized male bird in reproductive condition. Following collection, the semen can be used immediately for motility assays, or frozen for DNA extraction following the protocol described herein. This extraction protocol was refined for avian sperm and has been successfully used on samples collected from several passerine species (*Passer domesticus*, *Spizella passerina*, *Haemorhous mexicanus*, and *Turdus migratorius*) and one columbid (*Columba livia*).

Video Link

The video component of this article can be found at https://www.jove.com/video/55324/

[1] The material in this chapter was co-authored by Aurelia Kucera and Britt Heidinger. Aurelia Kucera had primary responsibility for collecting samples, data collection, data analysis, and composition of the first draft of this chapter. Britt Heidinger was the primary provider of funding for materials and assisted in revisions of this chapter. This publication can be found as "Kucera, A.C. and B.J. Heidinger. 2018. Avian Semen Collection by Cloacal Massage and Isolation of DNA from Sperm. Journal of Visualized Experiments, doi:10.3791/55324."

Introduction

Birds are ideal subjects for studies involving sperm quality and competition (Briskie et al. 1992), sperm telomere dynamics (Monaghan 2010), epigenetics (Carrell 2012), and similar topics, as they are widely used in biological research and sperm can be easily sampled using cloacal massage. Cloacal massage is a gentle, non-invasive manual technique for collecting semen from birds (Burrows and Quinn 1937; Wolfson 1952; Kast et al. 1998). Repeated samples can be obtained easily and no special tools are required, making it simple to perform in the field or lab. Although cloacal massage has been in use for decades, it is difficult to learn from the available written descriptions. This publication is intended to reduce the time and uncertainty involved in learning cloacal massage.

Semen collected from birds using cloacal massage can be used immediately for motility assessment (Burrows and Quinn 1937; Helfenstein et al. 2010) or artificial insemination (Purchase and Earle 2012), or frozen for other uses such as advanced imaging and DNA extraction. Semen samples from passerine birds are small but contain densely packed sperm. DNA is extracted using a commercial extraction kit for simplicity, with modifications to overcome the specialized protective features of sperm (Gill et al. 1985). After extraction, sperm telomeres can be measured using qPCR (Criscuolo et al. 2009).

Protocol

This protocol involves vertebrate animal subjects and has been approved by the Institutional Animal Care and Use Committee (IACUC) at North Dakota State University.

Semen collection from a passerine bird using cloacal massage

NOTE: Cloacal massage is an effective semen collection technique on reproductively active birds only, but can be performed successfully outside of the breeding season in

appropriate captive situations. Reproductive activity should be determined for target species prior to using cloacal massage. This technique can be used on wild birds upon capture or on birds held in captivity.

1. Starting with a male bird, (in breeding condition) bander's grip in the non-dominant hand, transfer the bird to the dominant hand with its ventral side touching the palm (Figure 2).
    a. Ensure that the bird's head is nearest to the lateral edge of the palm. Lightly secure the head and body using the pinky, ring, and middle fingers. This leaves the bird's vent and tail exposed.
    b. Leave the bird's legs loose or lightly restrained using the palm.

Figure 2. Grip for cloacal massage. The bird is secured lightly in the dominant hand with its ventral side touching the palm and head secured by the pinky finger.

2. Place the dominant index finger and thumb on either side of the superior end of the cloaca and lightly pinch the fingers together while applying very slight pressure toward the tail (Figure 3). The cloaca will evert, exposing the cloacal mucosa (pink interior). NOTE: If the bird is not currently producing semen, the clocal protuberance can be small and the

cloaca is difficult to evert. With experience, it is easy to differentiate between the cloacas of reproductively active and inactive males (Burrows and Quinn 1937; Lovette and Fitzpatrick 2016).

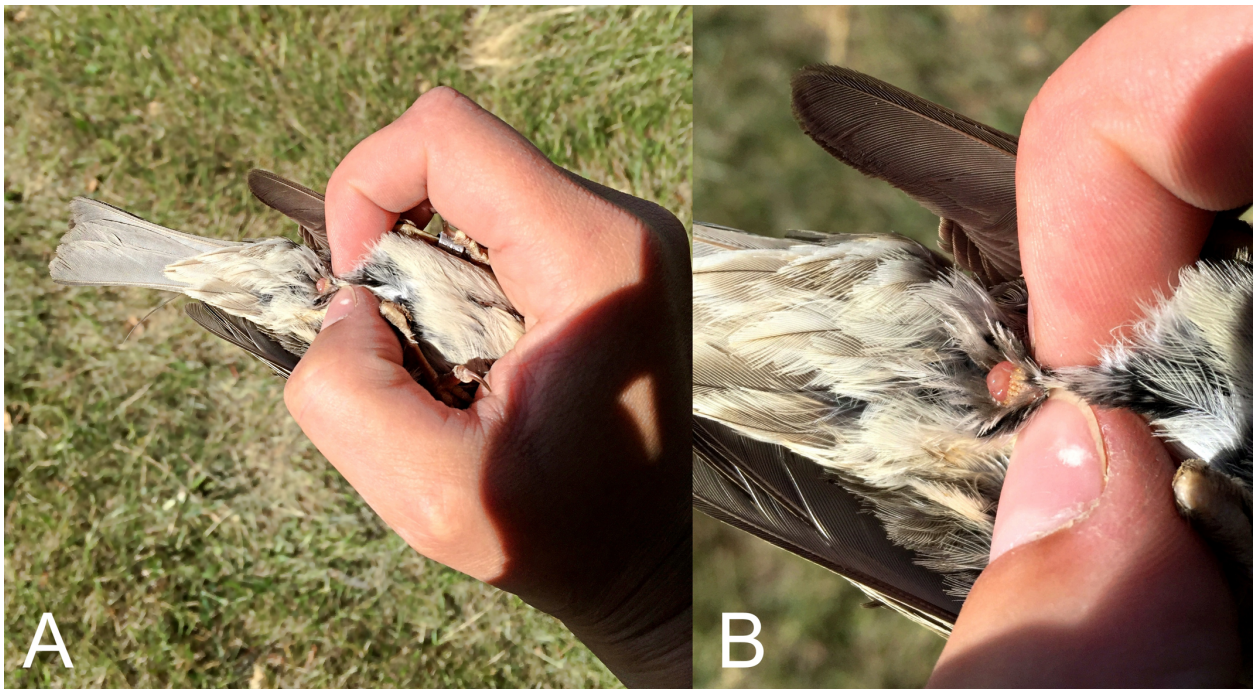

Figure 3. Finger position and cloacal protuberance. Evert the cloaca by applying slight pressure to the superior end (A). In a reproductively active male passerine, the cloacal protuberance is obvious and firm (B).

3. With the non-dominant hand, place the pad of the index finger on the dorsal side of the base of the tail to stabilize the hand, and then place the tip of the thumb just below the everted cloaca. NOTE: A short and smooth thumbnail is advantageous for performing cloacal massage and protects the bird from accidental injury.
4. Move the non-dominant thumb deep and cranially in a repetitive motion with medium pressure, staying below the cloaca. Continue until the bird ejaculates. Depending on the individual, this typically takes between 1 and several dozen strokes.
5. As soon as the bird ejaculates, collect the semen in a microhematocrit tube. Repeat cloacal massage to obtain multiple ejaculates. NOTE: Passerine semen in light brown and

typically has a somewhat thick consistency. White or dark substances ejected from the cloaca are not semen, and may contaminate the semen sample. The appearance of a small amount of blood on the cloaca or mixed in with the semen is usually caused by too much pressure during massage.

6. For DNA extraction, place the semen into a microcentrifuge tube with 20 μL 0f 1x PBS (pH 7.4). NOTE: Semen stored in 1x PBS is not suitable for artificial insemination, but can be used for DNA extraction at a later date when stored at -80 °C. The PBS can be room temperature or chilled, but should be in a liquid state when the semen sample is deposited.
   a. Confirm the presence of sperm in the sample by viewing a small amount of the diluted sample under a compound microscope (Figure 4).
7. Store the diluted semen at -80 °C until extraction.

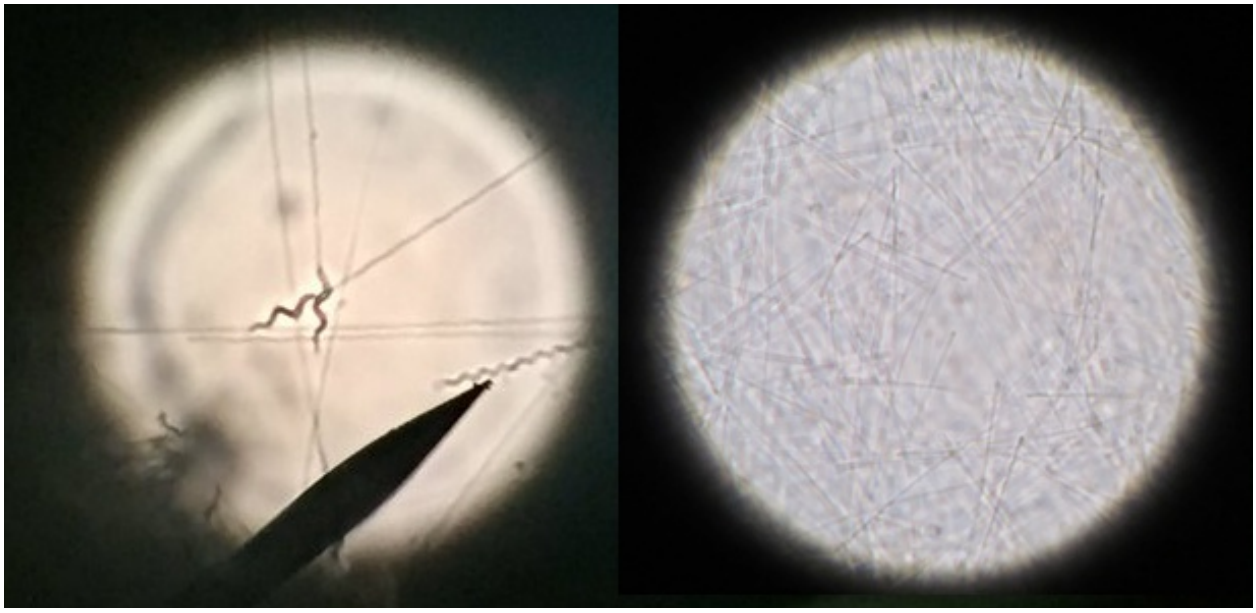

Figure 4. Sperm from two small passerines. These sperm samples were obtained by cloacal massage from a house finch (*Haemorhous mexicanus*), left, and a house sparrow (*Passer domesticus*), right, trapped in Fargo, ND in 2016. The samples were each diluted in 20 μL PBS and viewed under a compound microscope at 1,000X and 400X magnification, respectively.

Extraction of DNA from bird semen

NOTE: This protocol has been tested with multiple DNA extraction kits, but has only been successful when used with one kit (QIAamp® DNA Micro Handbook 2014). Steps that are modified from the kit protocol are indicated by *.

1. Prepare a 1 M solution of dithiothreitol (DTT) in water and mix using a vortex.*
    a. Aliquot the DTT solution and store it at -20 °C until immediately before use. Each sample to be extracted requires 10 µL of 1 M DTT solution. *
2. Prepare a buffer containing 20 mM Tris-CL (pH 8.0); 20 mM EDTA; 200 mM NaCL; and 4% SDS. Each sample to be extracted requires 90 µL of this buffer.*
    a. Store the buffer at room temperature. If it has precipitated, warm it to 56 °C to dissolve.*
3. Preheat an incubator containing a rocker or shaker to 65 °C.*
4. Immediately before beginning the extraction, mix 10 µL DTT solution with 90 µL of the buffer prepared in step 2b for each sample to be extracted, *e.g.* 900 µL buffer plus 100 µL DTT solution if extracting 10 samples. This mixture will be called DTT-buffer.*
5. Add 70 µL 1x PBS to the entire diluted semen sample and mix well using a vortex.*
6. Add 100 µL DTT-buffer and 10 µL proteinase K solution to the sample. Vortex for 20 seconds, then incubate in the preheated incubator for 1 hour on a rocker or shaker.*
7. Add 100 µL buffer AL and 200 µL fresh EtOH (100%) and vortex for 20 seconds.*
8. Transfer the entire sample to a spin column and centrifuge 1 minute at 6,000 x g. Discard the collection tube.
9. Place the column on a new tube and add 500 µL prepared buffer AW1. Centrifuge 1 minute at 6,000 x g. Discard the flow-through and place the column back on the tube.

10. Place the column on a new tube and add 500 µL prepared buffer AW2. Centrifuge for 1 minute at 6,000 x g. Discard the flow-through and place the column back on the tube.
11. Centrifuge 3 minutes at 20,000 x g. Discard the collection tube and place the column on a 1.5 mL microcentrifuge tube.
12. Pipet 35 µL of AE buffer directly onto the column membrane and incubate at room temperature for 5 minutes.*
13. Centrifuge 1 minute at 14,000 x g. Discard the spin column and store extracted samples at -80 °C.

Representative Results

Cloacal massage and DNA extraction using the described protocol has been performed on several passerine species and one columbid (Table 1). The presence of sperm in semen samples collected by cloacal massage was confirmed by viewing a small amount of the diluted samples under a compound microscope at 400X magnification (Figure 4). After extracting DNA from semen samples collected from house sparrows using the protocol described here, eight samples were run on gel electrophoresis with a DNA ladder (three samples can be seen in Figure 5). Distinct bands without smearing were seen in all eight of the sample lanes, indicating successful isolation of high quality DNA. All other samples extracted using this protocol were assessed for DNA concentration using a spectrophotometer (See Table 1).

Table 1. Species and samples collected and extracted to date (2016).

	House sparrow (*Passer domesticus)*	Chipping sparrow (*Spizella passerina*)	House finch (*Haemorhous mexicanus*)	American robin (*Turdus migratorius*)	Rock pigeon (*Columba livia*)
Number of samples collected	156	3	2	2	2
Number of samples extracted	90	3	2	2	2
Semen collection success rate	98%	75%	100%	67%	67%

At the time of publication (2016), samples have been processed using the described protocol from 4 passerine species and 1 columbid. Semen collection success rate refers to the percentage of each for which a sample was successfully obtained out of the total number attempted.

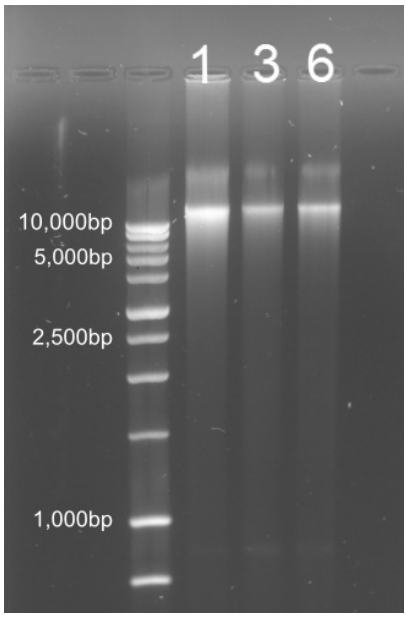

Figure 5. DNA gel electrophoresis of sperm DNA. Three samples of DNA extracted from house sparrow semen run on a gel with a DNA ladder. Note the presence of distinct bands, indicating successful extraction of high quality DNA.

Discussion

We describe a simple and reliable method for collecting semen from small and medium birds in reproductively active condition, and extracting DNA from avian sperm.

The described semen DNA extraction protocol is modified from a kit for simplicity, but was refined for use on avian semen. Sperm are resistant to lysis by standard extraction chemicals (Gill et al. 1985; Carter et al. 2000), and semen samples collected from passerines are fairly small (typically a few microliters). These factors present challenges that are successfully overcome by using an appropriate concentration of DTT and incubating at 65 °C. Using this method, we have consistently isolated usable amounts of DNA for qPCR analysis of sperm telomere length (typically between 40 - 250 ng/μL, depending on ejaculate volume).

DTT has previously been used to extract DNA from avian sperm in combination with a phenol/chloroform reaction (Carter et al. 2000). Phenol/chloroform reactions are effective for isolating DNA, but they are more time consuming, are more hazardous and require a fume hood. A notable limitation of our extraction protocol is that it has only been successful when used with one brand and type of extraction kit, possibly due to the small volume of starting sample. Very small semen samples (less than 1 μL) or samples with low sperm density may prevent successful extraction of DNA due to insufficient DNA present in the sample, but we have not encountered this problem when cloacal massage is performed on birds in breeding condition. To avoid this issue, cloacal massage can be repeated immediately to collect multiple ejaculates that can be pooled for extraction.

Cloacal massage has been in use for decades and in many species (Briskie and Montgomerie 1992; Burrows and Quinn 1937; Wolfson 1952; Kast et al. 1998; Helfenstein et al. 2010; Calhim et al. 2009; Samour et al. 1986; Stelzer et al. 2005), and is a quick and effective

means of collecting semen from birds in many cases. For studies involving wild or untrained birds that can be captured, it is more effective than collection methods that rely on convincing a male bird to copulate with an item and deposit his semen willingly (Burrows and Quinn 1937). Another semen collection method is by dissection of the testes or seminal glomera, but this is not an option for conservation purposes or repeated sampling (Burrows and Quinn 1937; Birkhead et al. 1997). The primary limitation of cloacal massage is the difficulty in learning the technique without demonstration. Written descriptions are often insufficient to understand and learn to implement the process. By illustrating cloacal massage in detail, we hope to reduce learning time and difficulty for those wishing to learn the technique.

Acknowledgements

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References

Birkhead, T.R., Buchanan, K.L., Devoogd, T.J., Pellatt, E.J. Székely, T., Catchpole, C.K. 1997. Song, sperm quality and testes asymmetry in the sedge warbler. Anim. Behav. 53:965-971.

Briskie, J.V., Montgomerie, R. 1992. Sperm size and sperm competition in birds. Proc. R. Soc. B. 247:89-95.

Burrows, W.D., Quinn, J.P. The collection of spermatozoa from the domestic fowl and turkey. 1937. Poultry Science 16(1):19-24.

Calhim, S., Immler, S., Birkhead, T.R. 2001. Sperm morphology and sperm velocity in passerine birds. Proc. R. Soc. B. 276:1175-1181.

Carrell, D.T. Epigenetics of the male gamete. 2012. Fertility and Sterility 97(2):267-274.

Carter, K.L., Robertson, B.C., Kempenaers, B. 2000. A differential DNA extraction method for sperm on the perivitilline membrane of avian eggs. Mol. Ecol. 9:2149-2154.

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N.B., Foote, C.G., Griffiths, K., Gault, E.A., Monaghan, P. 2009. Real-time quantitative PCR assay for measurement of avian telomeres. J. Avian Biol. 40:342-347.

Gill, P. Jeffreys, A.J., Werrett, D.J. 1985. Forensic application of DNA 'fingerprint.' Nature 318(12):577-579.

Helfenstein, F., Podevin, M., Richner, H. 2010. Sperm morphology, swimming velocity, and longevity in the house sparrow Passer domesticus. Behav. Ecol. Sociobiol. 64:557-565.

Kast, T.L., Ketterson, E.D., Nolan, V., Jr. 1998. Variation in ejaculate quality in dark-eyed juncos according to season, stage of reproduction, and testosterone treatment. Auk 115(3):684-693.

Lovette, I.J., Fitzpatrick, J.W. 2016. Cornell Lab of Ornithology Handbook of Bird Biology, 3rd Edition. Wiley-Blackwell, Princeton University Press. ISBN 9781118291047.

Monaghan, P. 2010. Telomeres and life histories: the long and short of it. Ann. N.Y. Acad. Sci. 1206:130-142.

Purchase, C.F., Earle, P.T. 2012. Modifications to the IMAGEJ computer assisted sperm analysis plugin greatly improve efficiency and fundamentally alter the scope of attainable data. J. Appl. Icthyol. 28:1013-1016.

QIAamp® DNA Micro Handbook. 2014. Third Edition.

Samour, J.H., Smith, C.A., Moore, H.D., Markham, J.A. 1986. Semen collection and spermatozoa characteristics in budgerigars (Melopsittacus udulates). Vet. Rec. 118:397-199.

Stelzer, G., Crosta, L., Bürkle, M., Krautwalt-Junghanns, M.E. 2005. Attempted semen collection using the massage technique and semen analysis in various psittacine species. J. Avian Med. Surg. 19(1):7-13.

Wolfson, A. 1952. The cloacal protuberance: a means for determining breeding condition in live male birds. Bird-Banding 23(4):159-165.

CHAPTER 3: SPERM TELOMERES, STRESS SENSITIVITY, AND OFFSPRING EARLY LIFE TELOMERE DYNAMICS: NATURAL VARIATION IN FREE-LIVING HOUSE SPARROWS

Abstract

Telomere dynamics is a mechanism that likely underlies aging in many taxa. Individuals of the same age differ in telomere length, and early life telomere length is predictive of longevity in birds. Stress is known to accelerate telomere loss, which may reduce longevity. Telomere length is usually measured in blood cells, and we have little knowledge of how telomere dynamics differ between tissues. Sperm is a tissue of particular interest as it has a hypothesized role in telomere length inheritance, but sperm may also be protected against telomere degradation. In a free-living population of house sparrows (*Passer domesticus*) we measured sperm and blood telomeres (n = 76), stress sensitivity (n = 60), and body condition in adult males. We also measured offspring early life telomere dynamics in nests for which we had blood and sperm samples from fathers (n = 15). Within individuals, blood and sperm telomere length were positively correlated, but sperm telomeres were longer than those in blood. Sperm telomere length varied across the breeding season. Blood telomere length was positively related to body condition, but this relationship was not found in sperm. There was no effect of stress sensitivity on telomere length in males. We found no relationship between offspring early life telomere dynamics and father's blood or sperm telomere length. These results suggest that sperm telomeres decline with age and may be sensitive to environmental factors, but are largely protected against degradation compared to blood telomeres. Our result of no relationship between father and offspring telomere length is based on a small sample size, and more studies

are needed to assess the potential link between paternal sperm telomere length near fertilization and offspring early life telomere dynamics.

Introduction

Understanding the mechanisms that contribute to variation in lifespan is of central importance to diverse fields including life history theory. Although the causes of aging are not fully understood, telomere dynamics (length and loss rate) is a potentially critical mechanism underlying longevity (Monaghan 2010; Monaghan 2014). Telomeres are highly conserved, non-coding regions of DNA at the ends of eukaryotic chromosomes. They shorten during cell division and telomere loss accelerates in response to stress, but telomere loss can be mitigated or reduced by the enzyme telomerase (Blackburn 2005; Monaghan and Haussmann 2006; Sahin and DePinho 2010; Monaghan 2014).

Telomere loss occurs throughout life due to accumulating oxidative damage and normal DNA replication processes (Monaghan 2014; Zhao et al. 2014; Haussmann and Heidinger 2015). Stress exposure has been shown to accelerate telomere loss and reduce longevity, particularly when stress is experienced in early life (Epel et al. 2004; Haussmann and Marchetto 2010; Tyrka et al. 2010; Herborn et al. 2014; Asghar et al. 2015). The impact of stress experience on telomere dynamics during adulthood is less studied, but there is evidence that adult stress can accelerate telomere loss in birds (Monaghan et al. 2012; Hau et al. 2015). However, mild stress can also increase longevity (Marasco et al. 2015). These hormetic effects may be the result of increased telomerase expression in response to stress (Monaghan 2014; Haussmann and Heidinger 2015). Elevation of circulating glucocorticoid hormones in response to stress, including the primary avian stress hormone corticosterone (CORT), leads to increased oxidative damage (Costantini et

al. 2011; Monaghan 2014). Therefore, individual differences in telomere loss rate may be partially mediated by differences in sensitivity to stress.

Telomere length varies between individuals of the same age, but early life telomere length is predictive of lifespan in birds, and adult telomere length can be predictive of future survival (Haussmann et al. 2005; Pauliny et al. 2006; Heidinger et al. 2012; Barrett et al. 2013). However, the inheritance of telomere length is not well understood (Monaghan 2010). In humans and some reptiles, offspring telomere length seems to be influenced by paternal age and telomere length (Njajou et al. 2007; Nordfjäll et al. 2010; Olsson et al. 2011). Evidence from birds suggests either primarily maternal inheritance or equal contribution of parents (Horn et al. 2011; Asghar et al. 2014; Atema et al. 2015; Reichert et al. 2015). However, it is unclear to what degree paternal effects influence early life telomere dynamics.

One hypothesized mechanism of telomere length inheritance is the direct transfer of gametic telomeres to zygotes (Graakjaer et al. 2004; Baird et al. 2006; Monaghan 2010). Telomere length in the germ line is maintained by telomerase, which is upregulated throughout life in avian gonads (Monaghan and Haussmann 2006; Haussmann et al. 2007; Liu et al. 2007). In spite of the high levels of telomerase, germ line telomeres can change over time; this variation has the potential to influence offspring telomere length (Kimura et al. 2008; Monaghan 2010; Ferlin et al. 2013). Gonadal tissue is highly proliferative, and sperm are very susceptible to oxidative damage, so telomere loss could be higher in sperm as hypothesized in other tissues with high cell division (Haussmann et al. 2007; Almbro et al. 2011; Losdat et al. 2011; Schmidt et al. 2016). Alternatively, sperm telomeres may be sufficiently protected by upregulated telomerase, or subject to screening by selective destruction of low-quality and short-telomere

cells (Kimura et al. 2008; Eisenberg et al. 2012; Ferlin et al. 2013; Haussmann and Heidinger 2015).

Important in evaluating the role of germ line telomere length in shaping offspring early life telomere length is an understanding of germ line telomere dynamics in general, which is still lacking (Monaghan 2010). Human studies suggest that sperm telomere length increases with age, while leukocyte telomere length decreases, and that older fathers produce offspring with longer telomeres (Kimura et al. 2008; Aston et al. 2012; Eisenberg et al. 2012; Ferlin et al. 2013; Yang et al. 2015). Beyond this, little is known about potential differences in telomere dynamics between tissues, partly due to the difficulty of obtaining repeat samples of many tissues (Reichert et al. 2013; Schmidt et al. 2016). To date, sperm telomere length has only been reported in humans, pigs, and cattle (Kozik et al. 1998; Ferlin et al. 2013).

We measured sperm telomere length in a free-living population of house sparrows in order to relate it to a) blood telomere length, b) body size and season, c) stress sensitivity, and d) offspring early life telomere dynamics. Based on preliminary data, we predicted a positive relationship between blood and sperm telomere length. With respect to offspring telomere length, we hypothesized a positive relationship with paternal sperm telomere length, similar to observed relationships in humans.

Methods

Study subjects

Adult male house sparrows were captured at our free-living nest box population on agricultural sites on North Dakota State University campus during the breeding seasons of 2014 through 2016. Birds were caught using mist nets and potter traps (non-targeted) or on the nest using custom nest box traps (targeted; Figure 6). In 2016, chicks from all nests in our population

were sampled for use in maintaining known-age population records and for various experiments. All procedures were approved by the IACUC at North Dakota State University (#A17035).

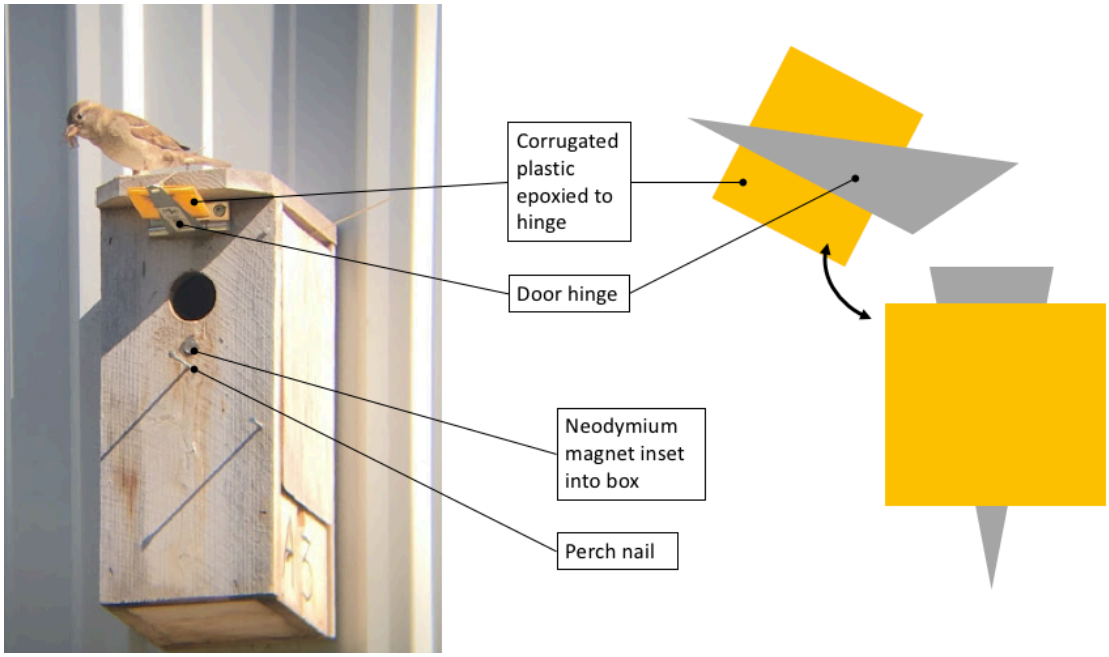

Figure 6. Our custom nest box trap (diagram and photo). The body of the trap is constructed from a square of 4mm corrugated plastic attached by epoxy to a 4" T-hinge, leaving the hole at the pointed end of the hinge unobstructed. The hinge is attached to the nest box above the entrance hole. A neodymium magnet is inset into the box below the hole, positioned to allow the hinge to cover the magnet when the trap door is closed. A light cord with a loop on the free end is tied to the hole at the end of the hinge. This loop is hooked to a finishing nail on the top of the nest box, keeping the trap door open most of the time. When the trap is to be used, the loop is removed from the nail and attached to a fishing line with a small trigger snap. The line is threaded over the finishing nail on top of the nest box, and reeled out (using a small fishing pole) to the blind, keeping enough tension on the line to hold the trap door in its normal (open) position. As soon as the target bird enters the nest box, tension is rapidly released from the line and the hinge swings closed, attaching to the magnet and preventing the bird's escape. This targeted trapping technique is often called bird fishing.

Blood and semen sampling

For adults, approximately 75 µL of whole blood was collected from the brachial vein into a heparinized micro-hematocrit tube within 3 minutes of capture. This sample was used for both telomere analysis (red blood cells) and baseline CORT measurement (plasma). At this time, morphometric measurements (mass, wing chord) and a semen sample was collected using

cloacal massage (Wolfson 1952; Kucera and Heidinger 2018). Semen was immediately diluted into 20 μL of 1x PBS and stored on ice for up to 8 hours until stored at -80 °C.

Most birds in 2016 were also subjected to a standardized capture-restraint protocol to measure sensitivity to stress. After the initial blood sample, the bird was placed in a soft cloth bag for 30 minutes, at which point a stress-induced blood sample (75 μL) was collected. Previous studies have shown that 30 minutes of restraint elicits maximal circulating CORT in house sparrows (Breuner and Orchinik 2001; Lindstrom et al. 2005). All blood samples were immediately stored on ice for up to 8 hours, centrifuged, separated into plasma and red blood cell fractions, and frozen at -80 °C until use.

Blood samples were collected from chicks twice during the nestling period. At 2 days of age (or when the majority of the chicks in the nest were 2 days of age), a very small (~5 μL) was collected from the brachial vein into a heparinized micro-hematocrit tube and immediately diluted in 20 μL of 1x PBS and stored on ice. At 10 days of age (or when the majority of the chicks in the nest were 10 days of age), samples of ~50-75 μL were collected and treated in the same way as adult samples. All blood samples were immediately stored on ice for up to 8 hours, centrifuged, separated into plasma and red blood cell fractions, and frozen at -80 °C until use.

Hormone quantification

A commercially available CORT enzyme immunoassay kit (Enzo Life Sciences ADI-901-097) was used for CORT analysis following a protocol previously validated in house sparrows (Kuhlman and Martin 2010; Martin et al. 2011). Briefly, 5 μL of 10% steroid displacement buffer was added to 5 μL of each plasma sample, 240 μL of assay buffer was added, and the sample mixture was aliquoted in duplicate to the assay plate. A standard curve ranging from 32 – 200,000 pg/ml was aliquoted in triplicate. All wells were incubated with

conjugated CORT and antibody on a shaker, wells were washed, and substrate was added before a second incubation. Stop solution was added, and the plate was read at 405 nm (corrected at 580 nm per manufacturer's recommendation). Inter- and intra-assay variation were 8.80% and 3.06% respectively.

Measurement of telomeres

DNA was extracted from red blood cells using commercially available extraction kits (Macherey-Nagel NucleoSpin Blood #740951) and following manufacturer protocol. DNA was extracted from whole diluted semen samples using commercially available extraction kits with modifications, following Kucera and Heidinger (2018). After extraction, DNA concentration and purity were assessed with a Nanodrop 8000 spectrophotometer (Thermo Scientific).

Relative telomere length was measured using quantitative PCR on a Strategene Mx3000P as described in Cawthon (2002) and modified for use in house sparrows. The relative telomere length of samples was calculated as the ratio (T/S) between telomere repeat copy number (T) and single copy control gene number (S) relative to the reference sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single copy control gene. The telomere forward and reverse primers used were: Tel 1B (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′), Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′). Zebra finch GAPDH primers were used to amplify the control gene: GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3'), GAPDH-R (5'CCATCAGCAGCAGCCTTCA-3'). The telomere and GAPDH reactions were run on separate plates, and the number of PCR cycles (C_t) required for the products to accumulate enough fluorescence to cross a threshold was determined each time. Samples with relatively low C_t values had longer telomeres than those

with relatively high C_t values. All samples were run in triplicate, and average values were used to determine the T/S ratio using the following formula: $2^{\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{telo} – C_t^{GAPDH})$ reference - $(C_t^{telo} – C_t^{GAPDH})$ (Agilent Technologies 2012).

The efficiency of each reaction was measured by including on each plate a standard curve produced by serial dilution of a reference sample. This sample was a pool of DNA from several female house sparrows collected in 2014. In all cases, plate efficiencies were within the accepted range of 100 ± 15%, and samples fell within the bounds of the standard curve. Intra-plate variation of Ct for GAPDH and telomere plates were 0.044% and 0.083% respectively, and inter-plate variation of ΔCt was 5.43%.

Statistical analysis

All T/S ratios and CORT values were natural-log transformed to ensure normality. To compare season effects between years, we used relative day of year (rDOY), calculated as the day of sampling minus the day of the first hatch in the population. Body condition was calculated as the residual of mass by wing chord; values above zero indicate relatively heavier birds, while values below zero indicate relatively lighter birds (Geiger et al. 2012). Pearson correlation and mixed models were used to determine the relationship between blood and sperm telomere length within individuals (n = 76), and between CORT and telomere length within individuals (n = 60). In the first mixed model, T/S ratio was the dependent variable, with tissue as a fixed effect and individual as a random effect. In the second set of mixed models, blood or sperm T/S ratio was the dependent variable, baseline and stress-induced CORT, rDOY, and body condition were fixed effects, and plate was a random effect. Two further mixed models were used to consider relationships between body condition, season, and year, since all birds with a CORT measure

were captured in 2016. Blood or sperm T/S was the fixed effect, body condition, rDOY, year, and rDOY x year were fixed effects, and plate was a random effect.

For a subset of nests, we had 2-day and 10-day nestling blood samples as well as the father's blood and semen samples collected during incubation or early nestling phase (n = 15). Mixed models were performed to determine the relationship between a father's blood and sperm telomere length and his offspring's early life telomere length. In one, the dependent variable was T/S ratio at day 2, with father's blood T/S ratio as a fixed effect, and nest and plate as random effects. A similar model was run with father's sperm T/S ratio as the fixed effect. In the next model, the dependent variable was T/S ratio at day 10, with father's blood T/S ratio as a fixed effect, and nest, plate, brood size, and offspring T/S ratio at day 2 as random effects. A similar model was run with father's sperm T/S ratio as the fixed effect. To determine whether chicks experienced change in telomere length between 2 and 10 days of age, a mixed model was used for all 2016 nestlings for which we had both samples (n = 160). With telomere length as the dependent variable, age was a fixed effect and individual and plate were included as random effects.

Results

Considering birds from all years, blood and sperm telomere length were positively correlated ($R^2 = .362$, $p = .0011$; Figure 7). Within individuals, telomere length in sperm was longer than in blood ($F_{1,74.99} = 110.15$, $p < .0001$; Figure 8). Cross-sectionally, sperm telomere length was significantly related to both season (rDOY; $F_{1,72.2} = 13.72$, $p = .0004$) and season by year ($F_{1,72.4} = 17.52$, $p < .0001$); sperm telomere length increased with date in 2014 but decreased with date in 2016 (Figure 9). There were no effects of season, year, or body condition on blood telomere length.

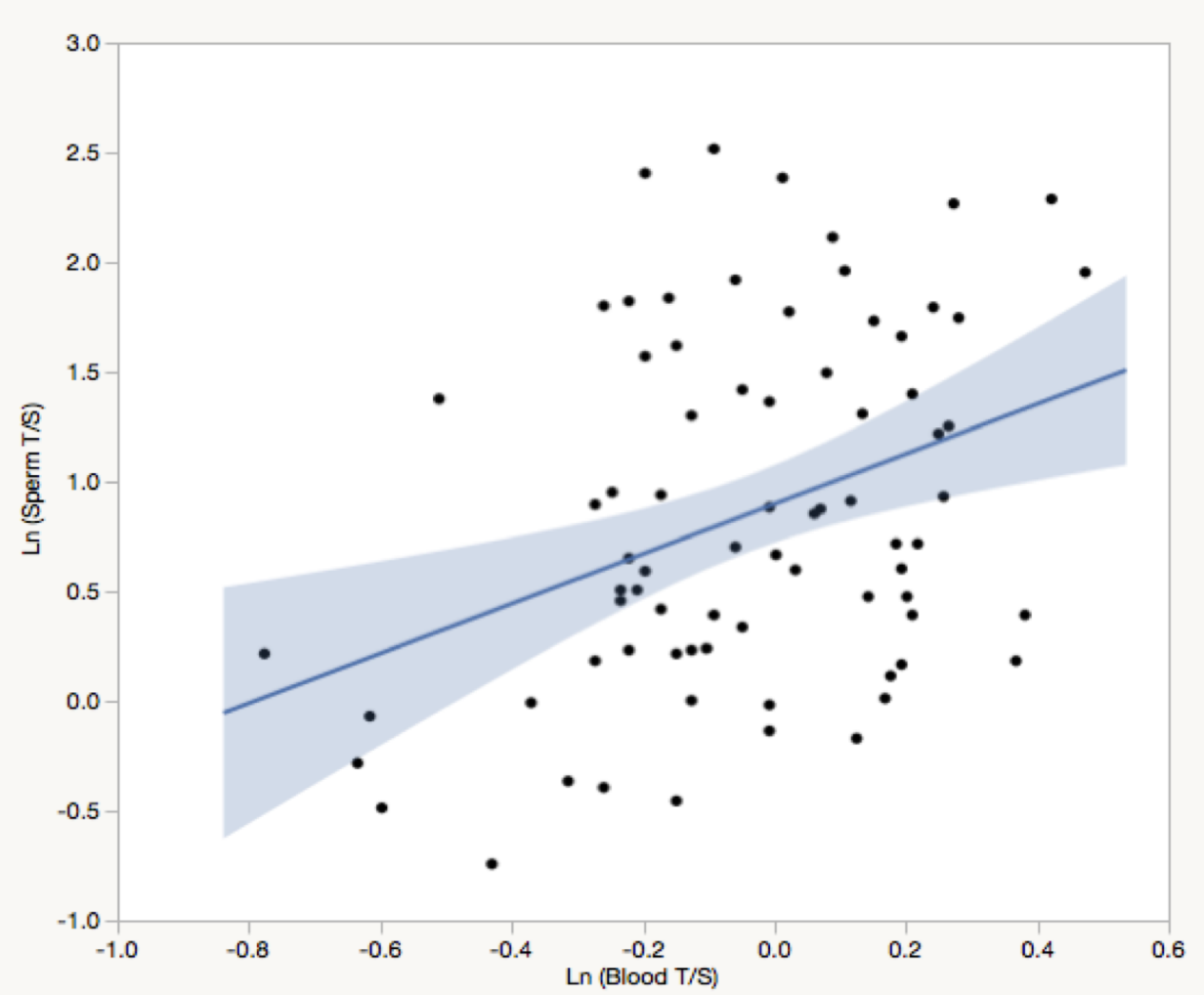

Figure 7. Correlation between blood telomere length and sperm telomere length in free-living male house sparrows ($R^2 = .362$, $p = .0011$).

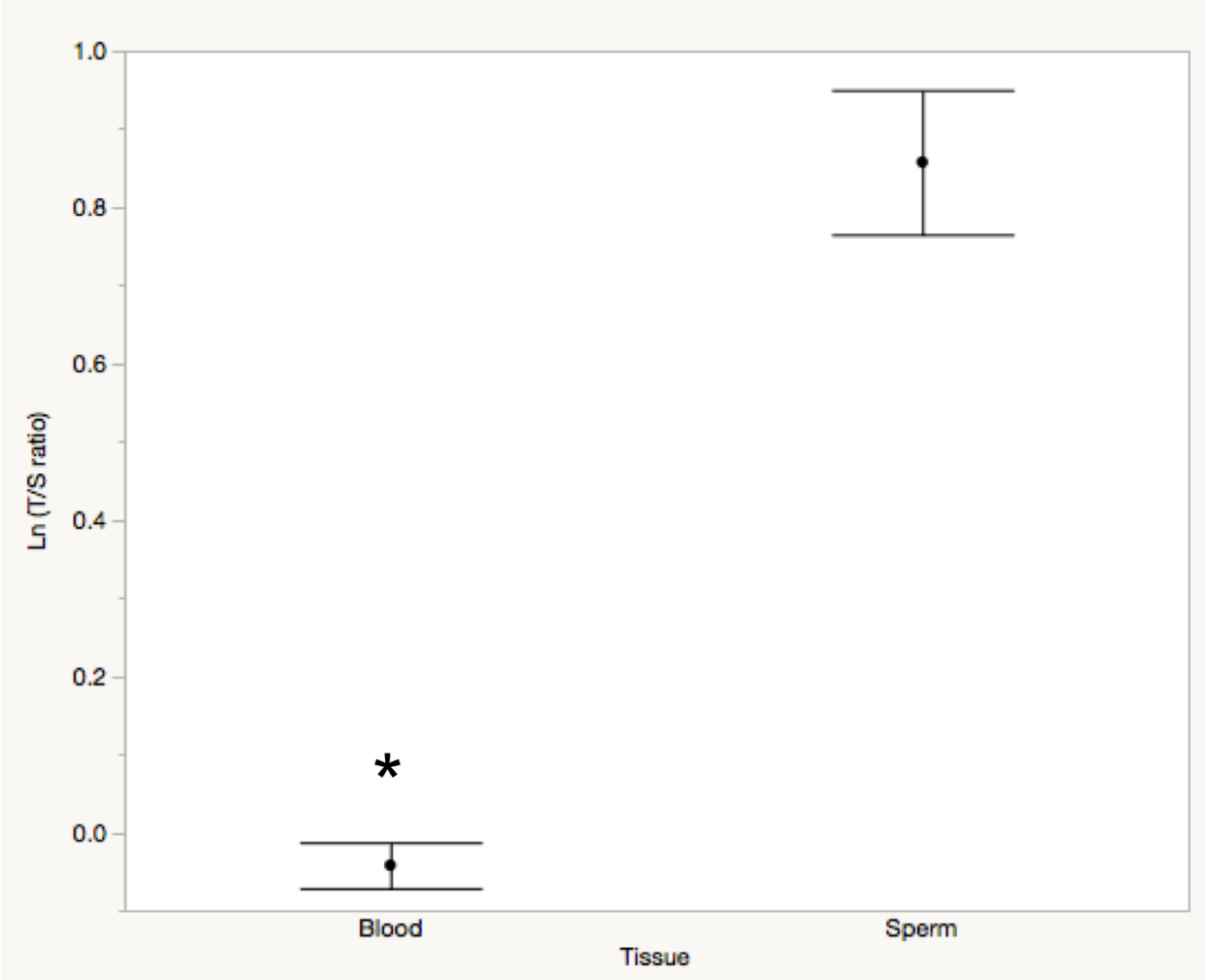

Figure 8. Mean and SEM of telomere length of red blood cells and sperm in free-living male house sparrows. Within individuals, sperm telomeres were significantly longer than blood telomeres ($p < .0001$).

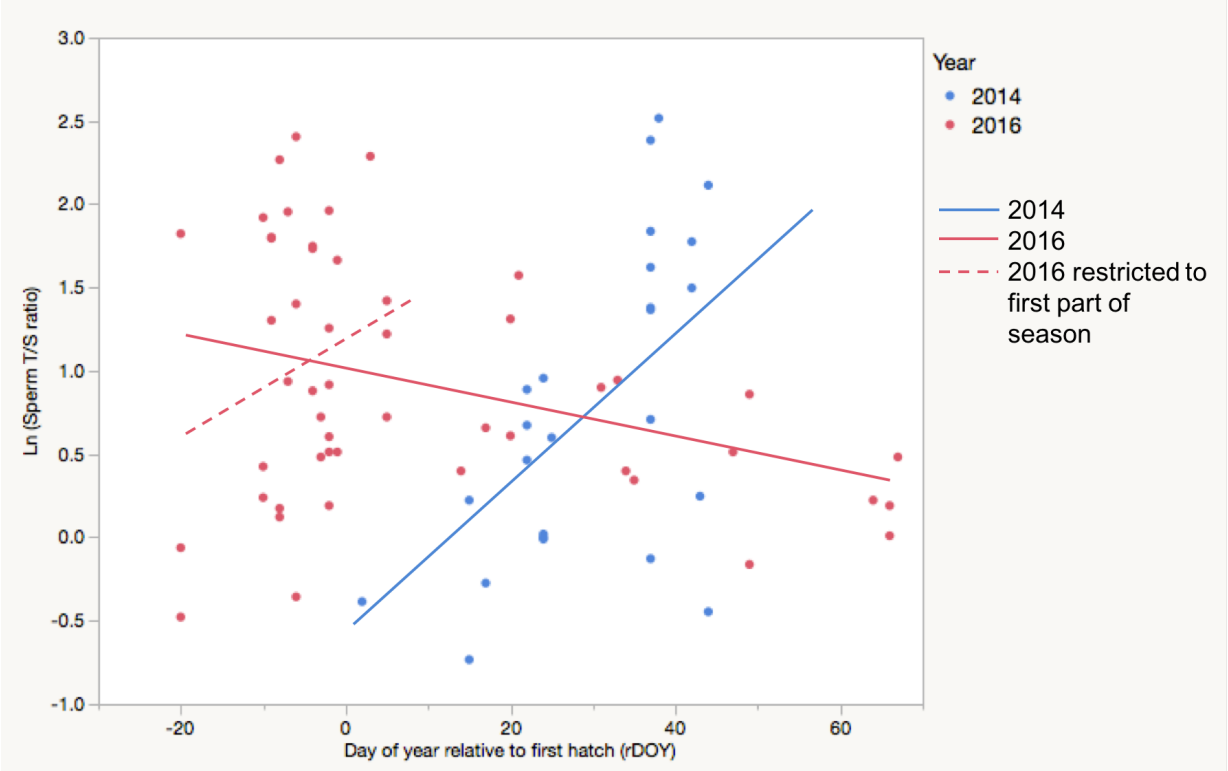

Figure 9. Correlation between season and sperm telomere length by year. Season is represented by day of year relative to the first hatch in the population (rDOY). The solid lines depict the relationship between sperm telomere length and season per year (season effect and season by year effect). The dashed line represents the relationship between sperm telomere length and season in 2016 if rDOY is restricted to include only the first wave of captures.

In 2016, body condition was predictive of blood telomere length so that birds with higher mass to wing chord ratios had longer telomeres ($F_{1,46.33} = 10.73$, $p = .002$), but body condition was not related to sperm telomere length. Baseline CORT, stress-induced CORT, and season were not related to either blood or sperm telomere length.

There was no effect of father's blood or sperm telomere length on offspring telomere length at either time point. There was no effect of age on telomere length in chicks when controlling for individual and plate ($F_{7,253.5} = 1.011$, $p = .423$).

Discussion

The role of telomere dynamics in mediating aging and stress-related fitness consequences has been a subject of increasing interest in recent years, but most studies consider only telomeres

in blood cells. In order to broaden our understanding of telomere dynamics in general and open the doors to exploring the relationship between germ line telomere dynamics and offspring early life telomere dynamics, this study measured blood and sperm telomeres, stress sensitivity, and nestling telomeres in a free-living population of house sparrows.

In the first report of sperm telomere length in birds, we found that within individuals, sperm telomeres were longer than blood telomeres, but the two were positively correlated. Contrary to findings in humans, this suggests that sperm and blood telomere length both decline with age in birds. That sperm telomere length exceeds blood telomere length may be related to the upregulated expression of telomerase in gonads, which could be investigated by measuring the within-individual relationship between testicular telomerase expression and sperm telomere length. Another possibility is that sperm telomeres are protected by gonadal dormancy for much of the year; during testicular regression, apoptosis of Sertoli and Leydig cells increases dramatically, and spermatogenesis is suppressed in the remaining spermatogonial stem cells (Young and Nelson 2001). Developing sperm are also subject to high rates of apoptosis during the breeding season, and those that have suffered oxidative damage may be targeted for destruction (Allen et al. 1987; Yin et al. 1998; Young and Nelson 2001).

Sperm telomere length varied across the season, and this variation differed between the two years of collection. The season by year effect is noticeable in Figure 9 as captures in 2014 were all after the first hatch and within a much shorter date range than 2016, but sperm telomere length increased across this period. Interestingly, the first wave of captures in 2016, which were mostly free captures (mist netted), shows a similar trend of increasing telomere length with date as in 2014. The second group of captures in 2016 were mostly targeted (nest box trapped), and this is where the decline in sperm telomere length with date is evident. While correcting for date

by using day of year relative to the population's first hatch does not ameliorate the season by year effect, it may be suggestive of a switch in sperm telomere dynamics with a change in breeding stage; targeted birds later in the 2016 season were known to have nestlings or eggs, while the free trapped birds (including all of 2014 captures) might not have been committed to parental care yet. Additionally, the nest boxes were newly installed in 2014 and a majority were unused. This potentially selected for younger or less desirable males being active in the vicinity of the boxes while trying to secure last-minute mates, shifting our view of sperm telomere length to those birds that were delayed in breeding compared to the general farm population. Finally, the weather was markedly different between the two years, which might have impacted the birds in numerous ways, from food availability and weather-related deaths to shifting onset of breeding (Marshall 1949; Wingfield and Kenagy 1991). In any case, it is clear that sperm telomere length is variable between individuals and across time (cross-sectionally). This supports the idea that sperm telomeres are sensitive to organismal or environmental factors, though to what degree sperm telomeres are damaged or protected remains an open question.

Blood telomere length did not show the same patterns of variation across the season or between years, but it was positively related to body condition. A similar result was found in young penguins, where lightweight individuals had had higher oxidative damage and shorter telomeres as compared to heavy birds (Geiger et al. 2012). Body condition is often used as an indicator of individual quality and may provide a longer-term view of individual ability to cope with adversity. Lighter birds may have higher oxidative stress leading to greater telomere attrition in a tradeoff between current and future survival.

Sperm telomere length was not related to baseline or stress-induced CORT levels in our population, but it is hard to interpret how this snapshot view relates to past stress exposure or

individual capacity for stress resistance. Longitudinal studies of sperm telomere dynamics, stress sensitivity, and oxidative load are needed to elucidate the relationship between stress and sperm telomere dynamics.

We found no evidence of a link between paternal telomere length and offspring early life telomere dynamics. Unfortunately, we had a small sample size of nests with paternal samples and feel that this question deserves further consideration. Human studies indicate that sperm telomere length is positively related to both offspring telomere length and zygote quality, while the relationship between parental and offspring blood telomere length in birds is unclear (Kimura et al. 2008; Asghar et al. 2014; Atema et al. 2015; Reichert et al. 2015; Yang et al. 2015). In light of the positive relationship between blood and sperm telomere length within individuals found here, future work would be enhanced by focusing on known-age individuals to uncover the relationship between paternal age, sperm telomere length, and offspring early life telomere length.

References

Agilent Technologies. 2012. Introduction to quantitative PCR.

Allen DJ, Harmon BV, Kerr JFR. 1987. Cell death in spermatogenesis. In: Potten CS, editor. Perspectives on Mammalian Cell Death. London: Oxford University Press. p. 229–258.

Almbro M, Dowling DK, Simmons LW. 2011. Effects of vitamin E and beta-carotene on sperm competitiveness. Ecol. Lett. 14:891–895.

Asghar M, Bensch S, Tarka M, Hansson B, Hasselquist D. 2014. Maternal and genetic factors determine early life telomere length. Proc. R. Soc. B Biol. Sci. 282:20142263.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science (80-.). 347:436–438.

Aston KI, Hunt SC, Susser E, Kimura M, Factor-litvak P, Carrell D, Aviv A. 2012. Divergence of sperm and leukocyte age-dependent telomere dynamics: Implications for male-driven evolution of telomere length in humans. Mol. Hum. Reprod. 18:517–522.

Atema E, Mulder E, Dugdale HL, Briga M, van Noordwijk AJ, Verhulst S. 2015. Heritability of telomere length in the Zebra Finch. J. Ornithol. 156:1113–1123.

Baird DM, Britt-Compton B, Rowson J, Amso NN, Gregory L, Kipling D. 2006. Telomere instability in the male germline. Hum. Mol. Genet. 15:45–51.

Barrett ELB, Burke TA, Hammers M, Komdeur J, Richardson DS. 2013. Telomere length and dynamics predict mortality in a wild longitudinal study. Mol. Ecol. 22:249–259.

Blackburn EH. 2005. Telomeres and telomerase: Their mechanisms of action and the effects of altering their functions. FEBS Lett. 579:859–862.

Breuner CW, Orchinik M. 2001. Seasonal regulation of membrane and intracellular corticosteroid receptors in the house sparrow brain. J. Neuroendocrinol. 13:412–420.

Cawthon RM. 2002. Telomere measurement by quantitative PCR. Nucleic Acids Res. 30:47e–47.

Costantini D, Marasco V, Møller AP. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181:447–456.

Eisenberg DTA, Hayes MG, Kuzawa CW. 2012. Delayed paternal age of reproduction in humans is associated with longer telomeres across two generations of descendants. Proc. Natl. Acad. Sci. 109:10251–10256.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. 101:17312–17315.

Ferlin A, Rampazzo E, Rocca MS, Keppel S, Frigo AC, De Rossi A, Foresta C. 2013. In young men sperm telomere length is related to sperm number and parental age. Hum. Reprod. 28:3370–3376.

Geiger S, Le Vaillant M, Lebard T, Reichert S, Stier A, Le Maho Y, Criscuolo F. 2012. Catching-up but telomere loss: Half-opening the black box of growth and ageing trade-off in wild king penguin chicks. Mol. Ecol. 21:1500–1510.

Graakjaer J, Pascoe L, Der-Sarkissian H, Thomas G, Kolvraa S, Christensen K, Londoño-Vallejo JA. 2004. The relative lengths of individual telomeres are defined in the zygote and strictly maintained during life. Aging Cell 3:97–102.

Hau M, Haussmann MF, Greives TJ, Matlack C, Costantini D, Quetting M, Adelman JS, Miranda AC, Partecke J. 2015. Repeated stressors in adulthood increase the rate of biological ageing. Front. Zool. 12:1–10.

Haussmann MF, Heidinger BJ. 2015. Telomere dynamics may link stress exposure and ageing across generations. Biol. Lett. 11:20150396.

Haussmann MF, Marchetto NM. 2010. Telomeres: Linking stress and survival, ecology and evolution. Curr. Zool. 56:714–727.

Haussmann MF, Winkler DW, Huntington CE, Nisbet ICT, Vleck CM. 2007. Telomerase activity is maintained throughout the lifespan of long-lived birds. Exp. Gerontol. 42:610–618.

Haussmann MF, Winkler DW, Vleck CM. 2005. Longer telomeres associated with higher survival in birds. Biol. Lett. 1:212–214.

Heidinger BJ, Blount JD, Boner W, Griffiths K, Metcalfe NB, Monaghan P. 2012. Telomere length in early life predicts lifespan. Proc. Natl. Acad. Sci. 109:1743–1748.

Herborn KA, Heidinger BJ, Boner W, Noguera JC, Adam A, Daunt F, Monaghan P. 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. Proc. R. Soc. B Biol. Sci. 281:20133151.

Horn T, Robertson BC, Will M, Eason DK, Elliott GP, Gemmell NJ. 2011. Inheritance of telomere length in a bird. PLoS One 6:1–5.

Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, Cupples A, Hunkin JL, Gardner JP, Lu X, et al. 2008. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 4:1–9.

Kozik A, Bradbury EM, Zalensky A. 1998. Increased telomere size in sperm cells of mammals with long termin (TTAGGG)n arrays. Mol. Reprod. Dev. 51:98–104.

Kucera AC, Heidinger BJ. 2018. Avian Semen Collection by Cloacal Massage and Isolation of DNA from Sperm. JoVE:e55324.

Kuhlman JR, Martin LB. 2010. Captivity affects immune redistribution to skin in a wild bird. Funct. Ecol. 24:830–837.

Lindstrom K, Hasselquist D, Wikelski M. 2005. House sparrows (Passer domesticus) adjust their social status position to their physiological costs. Horm. Behav. 48:311–320.

Liu L, Bailey SM, Okuka M, Muñoz P, Li C, Zhou L, Wu C, Czerwiec E, Sandler L, Seyfang A, et al. 2007. Telomere lengthening early in development. Nat. Cell Biol. 9:1436–1441.

Losdat S, Richner H, Blount JD, Helfenstein F. 2011. Immune activation reduces sperm quality in the great tit. PLoS One 6.

Marasco V, Boner W, Heidinger B, Griffiths K, Monaghan P. 2015. Repeated exposure to stressful conditions can have beneficial effects on survival. Exp. Gerontol. 69:170–175.

Marshall AJ. 1949. Weather factors and spermatogenesis in birds. J. Zool. 119:711–716.

Martin LB, Kidd L, Liebl AL, Coon CAC. 2011. Captivity induces hyper-inflammation in the house sparrow (Passer domesticus). J. Exp. Biol. 214:2579–2585.

Monaghan P. 2010. Telomeres and life histories: The long and the short of it. Ann. N. Y. Acad. Sci. 1206:130–142.

Monaghan P. 2014. Organismal stress, telomeres and life histories. J. Exp. Biol. 217:57–66.

Monaghan P, Haussmann MF. 2006. Do telomere dynamics link lifestyle and lifespan? Trends Ecol. Evol. 21:47–53.

Monaghan P, Heidinger BJ, D'Alba L, Evans NP, Spencer KA. 2012. For better or worse: reduced adult lifespan following early-life stress is transmitted to breeding partners. Proc. R. Soc. B Biol. Sci. 279:709–714.

Njajou OT, Cawthon RM, Damcott CM, Wu S, Ott S, Garant MJ, Blackburn EH, Mitchell BD, Shuldiner AR, Hsueh W. 2007. Telomere length is paternally inherited and is associated with parental lifespan. Proc. Natl. Acad. Sci. U. S. A. 104:12135–9.

Nordfjäll K, Svenson U, Norrback K-F, Adolfsson R, Roos G. 2010. Large-scale parent-child comparison confirms a strong paternal influence on telomere length. Eur. J. Hum. Genet. 18:385–9.

Olsson M, Pauliny A, Wapstra E, Uller T, Schwartz T, Blomqvist D. 2011. Sex differences in sand lizard telomere inheritance: Paternal epigenetic effects increases telomere heritability and offspring survival. PLoS One 6.

Pauliny A, Wagner RH, Augustin J, Szep T, Blomqvist D. 2006. Age-independent telomere length predicts fitnessin two bird species. Mol. Ecol. 15:1681–1687.

Reichert S, Criscuolo F, Verinaud E, Zahn S, Massemin S. 2013. Telomere length correlations among somatic tissues in adult zebra finches. PLoS One 8:1–7.

Reichert S, Rojas ER, Zahn S, Robin J-P, Criscuolo F, Massemin S. 2015. Maternal telomere length inheritance in the king penguin. Heredity (Edinb). 114:10–16.

Sahin E, DePinho RA. 2010. Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 464:520–528.

Schmidt JE, Sirman AE, Kittilson JD, Clark ME, Reed WL, Heidinger BJ. 2016. Telomere correlations during early life in a long-lived seabird. Exp. Gerontol. 85:28–32.

Tyrka AR, Price LH, Kao H, Marsella SA, Carpenter LL. 2010. Childhood maltreatment and telomere shortening: Preliminary support for an effect of early stress on cellular aging. Biol. Psychiatry 67:531–534.

Wingfield JC, Kenagy JG. 1991. Natural regulation of reproductive cycles. In: Pang P, Schreibman M, editors. Vertebrate Endocrinology: Fundamental and Biomedical Implications. New York: Academic Press. p. 303–342.

Wolfson A. 1952. The Cloacal Protuberance : A Means for Determining Breeding Condition in Live Male Passerines. Bird-Banding 23:159–165.

Yang Q, Zhao F, Dai S, Zhang N, Zhao W, Bai R, Sun Y. 2015. Sperm telomere length is positively associated with the quality of early embryonic development. Hum. Reprod. 30:1876–1881.

Yin Y, Stahl BC, DeWolf WC, Morgentaler A. 1998. p53-mediated germ cell quality control in spermatogenesis. Dev. Biol. 204:165–171.

Young KA, Nelson RJ. 2001. Mediation of seasonal testicular regression by apoptosis. Reproduction 122:677–685.

Zhao Z, Pan X, Liu L, Liu N. 2014. Telomere length maintenance, shortening, and lengthening. J. Cell. Physiol. 229:1323–1329.

CHAPTER 4: NO EFFECT OF ACUTE IMMUNE CHALLENGE ON SPERM TELOMERE DYNAMICS IN CAPTIVE HOUSE SPARROWS

Abstract

Exposure to stressful events, including immune activation, is known to accelerate telomere loss in birds, likely due to increased oxidative damage. Stress experience in the parental generation may also impact telomere length in offspring, and one potential mechanism is direct transfer of gamete telomeres. However, little is known about the sensitivity of sperm telomeres to organismal stress exposure. In this study, we found that sperm telomere dynamics was not impacted by acute immune activation by exposure to LPS at short (24- and 48-hour) or moderate (14-day) time scales. We also found that sperm telomere length is weakly repeatable across the same time scales, while erythrocyte telomere length is highly repeatable across a relatively long period in a short-lived bird (6 months). This suggests that sperm telomere length is susceptible to change over a relatively short period, and sperm telomere loss may occur in response to organismal stress that is severe enough to overcome any sperm-protective mechanisms.

Introduction

Exposure to stressors can have lifelong fitness consequences, including accelerated aging and reduced longevity (Monaghan and Haussmann 2006; Monaghan et al. 2012; Monaghan 2014; Asghar et al. 2015). Although the causes of aging are not fully resolved, telomere dynamics (length and loss rate) is a potentially critical mechanism underlying longevity and therefore fitness (Monaghan 2010; Monaghan 2014). In support of this, early life telomere length is predictive of longevity in birds (Heidinger et al. 2012), but little is known about the inheritance of telomere length.

Correlational studies in humans have found relationships between parental and offspring telomere length, and between offspring telomere length and parental age at conception (Njajou et al. 2007; Kimura et al. 2008; Nordfjäll et al. 2010; Eisenberg et al. 2012; Monaghan 2014). In humans, these relationships may be stronger between fathers and children than between mothers and children (Nordfjäll et al. 2010). In birds, there is some evidence for maternal inheritance of telomere length, but it is unclear whether paternal effects also influence early life telomere dynamics (Horn et al. 2011; Asghar et al. 2014; Atema et al. 2015; Reichert et al. 2015).

One mechanism that could potentially contribute to telomere length inheritance is the direct transfer of gamete telomeres to zygotes (Graakjaer et al. 2004; Baird et al. 2006; Monaghan 2010). Telomere length in the germ line is maintained by the enzyme telomerase, which is upregulated throughout life in the gonads in birds (Monaghan and Haussmann 2006; Haussmann et al. 2007; Liu et al. 2007). In spite of this, germ line telomere length can change over time, and this variation has the potential to influence offspring telomere length (Kimura et al. 2008; Monaghan 2010; Ferlin et al. 2013).

Telomere loss occurs constantly throughout life due to accumulating oxidative damage and, to a lesser extent, normal DNA replication (Monaghan 2014; Zhao et al. 2014; Haussmann and Heidinger 2015). Stress exposure has been shown to accelerate telomere loss and negatively impact longevity, and the effects of stress can even be transmitted to offspring and further removed generations (Epel et al. 2004; Monaghan 2014; Haussmann and Heidinger 2015). This accelerated telomere loss is likely caused by increased oxidative damage in response to survival-promoting processes such as activation of the immune system and the stress axis (Monaghan 2014; Asghar et al. 2015; Haussmann and Heidinger 2015).

Activation of the immune system is a challenge experienced by many animals in the wild, which incurs costs similar to those observed during activation of the stress axis, including increased oxidative stress, increased metabolic rate, and energetic trade-offs (Norris and Evans 2000; Bonneaud et al. 2003; Needham et al. 2017). Like other stressors used in studies of telomere dynamics (including exogenous corticosterone, food restriction, handling, and increased reproductive effort), pathogen exposure can lead to telomere attrition in wild birds (Monaghan and Haussmann 2006; Beaulieu et al. 2011; Heidinger et al. 2012; Monaghan et al. 2012; Herborn et al. 2014; Asghar et al. 2015).

Gonadal tissue expresses telomerase throughout life in birds, whereas expression decreases after development in bone marrow and other somatic tissues in short-lived birds (Haussmann et al. 2007; Ferlin et al. 2013). Sperm are highly susceptible to oxidative damage, but this upregulated telomerase expression in the highly proliferative gonad may protect gamete telomeres against damage during times of increased oxidative stress, and telomerase expression may even increase in response to stress (Almbro et al. 2011; Losdat et al. 2011; Haussmann and Heidinger 2015). Additionally, gametes with short telomeres may be subject to selective destruction, so that telomere attrition during spermatogenesis could lead to no observed effect on telomere length in ejaculated sperm, or even an observed increase in mean telomere length (Kimura et al. 2008; Eisenberg et al. 2012; Ferlin et al. 2013). However, it is unclear to what degree these mechanisms could buffer the effects of stress on sperm telomeres.

Multiple pathways may mediate the transfer of the effects of stress to offspring, including direct (inheritance of germ line telomeres) and indirect pathways (such as parental care; Haussmann and Heidinger 2015). If exposure to stressors affects gamete telomere length, these effects could directly impact offspring telomere length at fertilization. Stress exposure may

influence telomeres in the male germ line at various time scales including: with age (long-term), during spermatogenesis (short-term), and near sperm maturation (very short-term).

To evaluate the potential transfer of paternal stress effects to offspring telomere dynamics via direct effects of sperm telomeres, we first need to understand the sensitivity of sperm telomere dynamics to organismal stress exposure. Sperm telomeres are likely to be sensitive to change on a shorter time scale than the telomeres of red blood cells because they are generated rapidly and have a high turnover rate (Rodnan et al. 1956; Amir et al. 1973).

We examined the effects of stress exposure on sperm telomere dynamics by exposing captive birds to acute, transient immune activation. While it was unclear whether we would observe attrition in sperm telomeres following a transient challenge, we predicted that acute attrition would not be observed at all sampling points, as sperm may be more sensitive to DNA damage at certain developmental points (i.e. spermatocytes vs. spermatids vs. mature spermatozoa). We also measured the repeatability of telomere length in both sperm and red blood cells.

We exposed captive male house sparrows (*Passer domesticus*) to a simulated immune challenge by injection with lipopolysaccharide (LPS). LPS is a component of the cell membrane of gram-negative bacteria that is targeted by the vertebrate immune system, leading to activation of the acute phase immune response without actual pathogen exposure (Dunn and Wang 1995; Turnbull et al. 1998; Bonneaud et al. 2003). In birds, LPS exposure induces increased oxidative stress and multiple sickness responses, including hyper- or hypothermia, anorexia, and behavioral changes (Kelley et al. 2003; Dantzer and Kelley 2007; Costantini 2008; Needham et al. 2017). In house sparrows specifically, LPS exposure has been shown to activate the acute phase immune response and stress-induced levels of corticosterone, and sustained captivity both

increased the immune response and decreased clearance rate of the endotoxin (Lee et al. 2005; Martin et al. 2011).

Methods

Study subjects

Mist nets and potter traps were used to capture 14 adult male house sparrows around Fargo, ND in October 2014. Birds were individually housed in 23.5-inch x 15.5-inch wire cages in one room at North Dakota State University for 6 months. Each cage was visually isolated using opaque plastic barriers on the sides and top. Food (Kaytee Supreme mixed seed for finches), water, and blue grit were provided *ad libitum*, and vitamin water was provided once per month (eCOTRITION Pro Ultra-Care Vita-Sol for caged birds).

Following capture, birds were kept on a short-day light cycle of 8L:16D for eight weeks to ensure photosensitivity (King and Farner 1963; Farner et al. 1966). The light cycle was then changed to long-day (16L:8D) to photostimulate the birds and trigger gonadal recrudescence (King and Farner 1963; Murton et al. 1970; Small et al. 2007). During the breeding season in Fargo, ND, free-living sparrows experience a maximal day length of 15 hours and 53 minutes, closely matching the experimental long-day light cycle (U.S. Naval Observatory). The room temperature was maintained between 22.2-23.9 °C for the duration of captivity. Animal care and experimental procedures were approved by the IACUC at North Dakota State University (#A14044).

Immune challenge

Birds were randomly assigned to either experimental or control treatment upon capture. In February 2015, experimental males (n = 7) received a 2.0 mg/kg intramuscular injection of lipopolysaccharide (LPS, from *E. coli* 055:B5, Sigma #L2880) diluted in PBS (Adelman et al.

2010; Sköld-Chiriac et al. 2014). Control males received an equal mass-calculated volume injection of 1x phosphate-buffered saline (PBS). All injections were given immediately following baseline semen collection (see below).

Blood and semen sampling

Upon initial capture and again at the end of the study, approximately 100 μL blood samples were collected from the brachial vein into heparinized micro-hematocrit tubes. Blood samples were stored on ice for less than 1 hour, centrifuged, separated into plasma and red blood cell fractions, and frozen at -80 °C until DNA extraction.

Semen samples were collected at 4 time points throughout the study: immediately prior to the experimental injection, and post-treatment at 24 hours, 48 hours, and 14 days. These post-treatment sampling points were chosen to capture potential effects on telomeres of mature sperm (replaced every 24 hours) and during early (14 day sample) and late (48 hour sample) spermatogenesis, which is thought to take 11-13 days in birds (Amir et al. 1973; de Reviers 1975; Lin and Jones 1992). Sperm samples were collected using cloacal massage (Wolfson 1952; Kucera and Heidinger 2018). Ejaculated semen was collected in 5 μL non-heparinized capillary tubes (Wheaton, Millville, NJ, USA, product #851321), and each semen sample was immediately diluted into 20 μL of 1x PBS and stored on ice for less than 10 minutes until frozen and stored at -80 °C.

Measurement of telomeres

DNA was extracted from red blood cells using commercially available extraction kits (Macherey-Nagel NucleoSpin Blood #740951) and following manufacturer protocol. DNA was extracted from whole diluted semen samples using commercially available extraction kits with

modifications, following Kucera and Heidinger (2018). After extraction, DNA concentration and purity were assessed with a Nanodrop 8000 spectrophotometer (Thermo Scientific).

Relative telomere length was measured using quantitative PCR on a Strategene Mx3000P as described in Cawthon (2002) and modified for use in house sparrows. The relative telomere length of samples was calculated as the ratio (T/S) between telomere repeat copy number (T) and single copy control gene number (S) relative to the reference sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single copy control gene. The telomere forward and reverse primers used were: Tel 1B (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′), Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′). Zebra finch GAPDH primers were used to amplify the control gene: GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3'), GAPDH-R (5'CCATCAGCAGCAGCCTTCA-3'). The telomere and GAPDH reactions were run on separate plates, and the number of PCR cycles (C_t) required for the products to accumulate enough fluorescence to cross a threshold was determined each time. Samples with relatively low C_t values had longer telomeres than those with relatively high C_t values. All samples were run in triplicate, and average values were used to determine the T/S ratio using the following formula: $2^{\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{telo} – C_t^{GAPDH})$ reference - $(C_t^{telo} – C_t^{GAPDH})$ (Agilent Technologies 2012).

The efficiency of each reaction was measured by including on each plate a standard curve produced by serial dilution of a reference sample. This sample was a pool of DNA from several female house sparrows collected in 2014. In all cases, plate efficiencies were within the accepted range of 100 ± 15%, and samples fell within the bounds of the standard curve. Intra-plate

variation of Ct for GAPDH and telomere plates were 1.18% and 1.78% respectively, and inter-plate variation of ΔCt was 18.7%.

Statistical analysis

All T/S ratios were natural log transformed to ensure normality. Linear mixed-effects models were conducted in SPSS (IBM SPSS 23) to examine the relationship between sperm and blood telomere dynamics, treatment, and sampling time point. Bird identity was included as a random effect to control for repeated measures, and blood and sperm sampling time point and treatment were included as fixed effects. A linear mixed-effects model was used to test the relationship between blood and sperm telomere length with telomere length as the dependent variable, tissue as a fixed effect, and bird as a random effect. Repeatability estimates for telomere length within tissues were calculated using the rptR package in R 3.4.2.

Results

Telomere dynamics

There were no significant effects of treatment, time point, or treatment by time point on sperm telomere length (treatment: $F_{1,47.29} = .169$, $p = .682$; time point: $F_{3,47} = .729$, $p = .154$; interaction: $F_{3,47} = .001$, $p = .605$, Figure 10). Within individuals, pre-treatment blood telomere length and sperm telomere length were positively correlated ($R^2 = 0.42$, $p = .012$, Figure 11). On average, an individual's sperm telomeres were not significantly different in length from his blood telomeres ($F_{3,37.7} = 2.4$, $p = .083$).

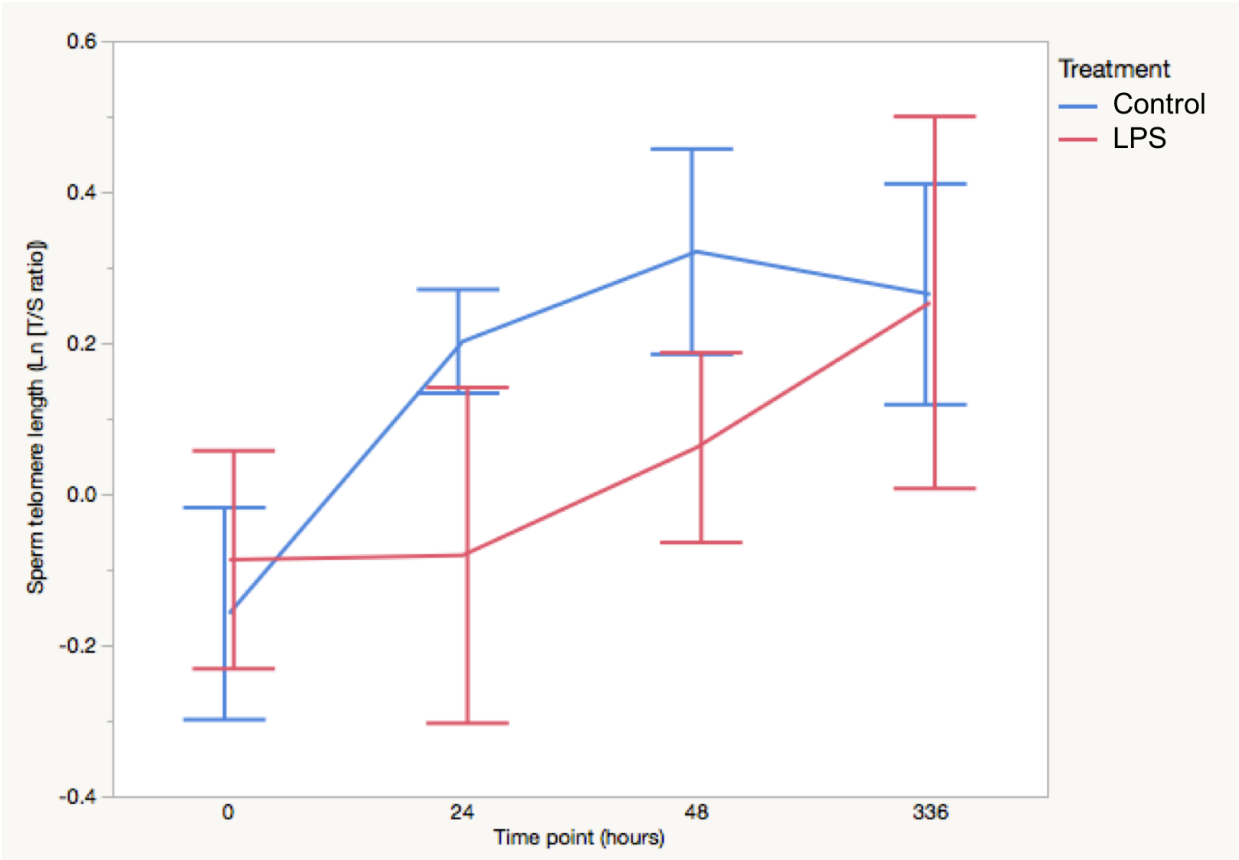

Figure 10. Sperm telomere length (mean and SEM) collected at four time points. LPS (n = 7, red) or vehicle (n = 7, blue) was injected into each bird immediately after semen collection at time point 0.

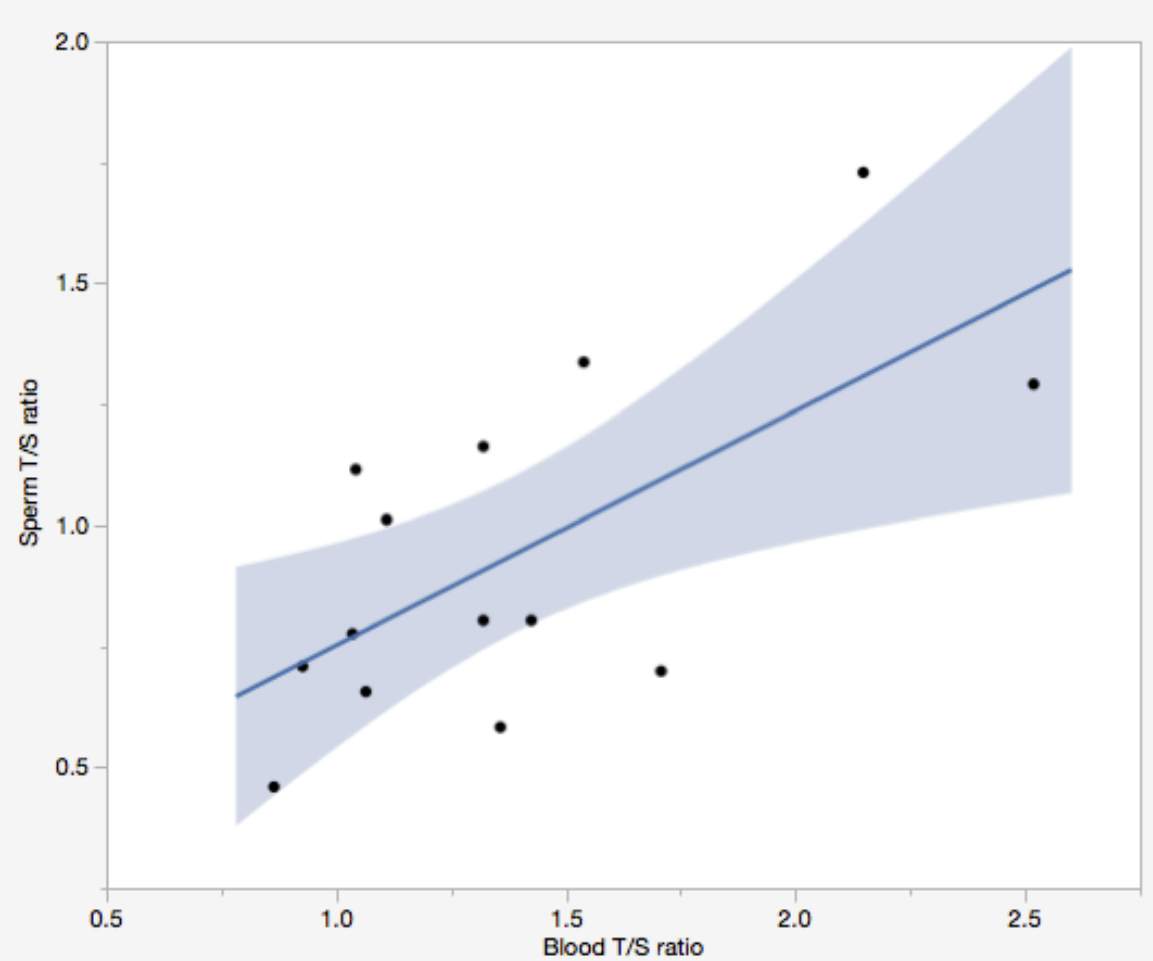

Figure 11. Relationship between blood and sperm relative telomere length (T/S ratio from qPCR) in captive house sparrows (n = 14, R^2 = .42, p = .012).

Repeatability of telomere length

Within individuals, blood telomere length was highly repeatable (r = 0.797, p = .0002) between 2 samples taken 6 months apart. Sperm telomere length was weakly repeatable (r = 0.223, p = .048) between 4 samples taken across a 14-day period.

Discussion

Exposure to stressful events, including immune activation, is known to accelerate telomere loss in birds, likely due to increased oxidative damage (Costantini et al. 2011; Monaghan 2014). Stress experience in the parental generation may also impact telomere length in offspring, and one potential mechanism is direct transfer of gamete telomeres (Graakjaer et al.

2004; Baird et al. 2006; Monaghan 2010). However, little is known about the sensitivity of sperm telomeres to organismal stress exposure. In this study, we found that sperm telomere dynamics was not impacted by acute immune activation by exposure to LPS at short (24- and 48-hour) or moderate (14-day) time scales. We also found that sperm telomere length is weakly repeatable across the same time scales, while erythrocyte telomere length is highly repeatable across a relatively long period in a short-lived bird (6 months).

In testing the sensitivity of sperm telomeres in response to acute immune activation, we found no relationship between sperm telomere dynamics and treatment, time point, or the interaction thereof. These birds did display patterns of acute phase immune response, including hypothermia and reduced circulating testosterone after LPS injection (see Needham et al. 2017), and previous studies have found that LPS causes acute phase response and increased metabolic rate in house sparrows (Martin et al. 2011; King and Swanson 2013). It is unclear whether hypothermia, like hyperthermia (fever response), is an adaptive component of immune activation, but house sparrows and other small passerines also display hypothermia in response to LPS (Owen-Ashley et al. 2006; Owen-Ashley and Wingfield 2007; King and Swanson 2013). Additionally, systemic hypothermia may protect the testes from heat damage when birds are exposed to immune challenges during the breeding season, especially given that avian testes do not have a mechanism for specialized temperature regulation like mammalian (external) testes (Waites 2012).

Considering the prevalence of hypothermia in small passerines, it seems likely that our sparrows did mount an immune response to LPS injection complete with upregulated metabolic rate, and therefore, increased oxidative stress. The fact that sperm telomere dynamics was not impacted by immune activation at any measured time point suggests that either sperm are

protected or that this acute challenge was not strong enough to induce a trade-off. If there are protective mechanisms to mitigate the effects of oxidative damage on gametes, they may include upregulated telomerase expression in the gonads (and potentially greater upregulation in response to stress) and selective destruction of sperm with short telomeres. Birds are frequently exposed to situations requiring acute activation of stress response or immune response during the breeding season, leading to a tradeoff between reproduction and survival (Wingfield and Sapolsky 2003). Resistance to stress during the breeding season (i.e. allocating resources toward reproduction) is often critical to reproductive success in passerines, which have short breeding seasons and short lifespans (Richner and Tripet 1999; Ardia 2005; Clark and Martin 2007). Our results may be consistent with house sparrows favoring protection of sperm in response to stress when testes are active in order to maximize lifetime reproductive success.

Alternatively, exposure to LPS may have been a mild enough stressor that the birds were able to cope with mounting a transient immune response at no cost to normal functions. Captivity is already stressful to house sparrows, and the additional challenge of immune activation may have been too minor to cause a noticeable difference in sperm telomere length between treatment and control individuals (Martin et al. 2011). Additionally, house sparrows are known to experience fewer costs of immune challenge compared to a non-invasive congener, indicating that they may be more resistant overall to stress or immune activation (Lee et al. 2005). In this case, birds were fed ad lib. and received a vitamin supplement, and may have had high enough antioxidant capacity that it was not overcome by an increase in reactive oxygen species due to LPS (Almbro et al. 2011). Indeed, LPS treatment also had no impact on sperm quality in these birds (see Needham et al. 2017), supporting the idea that the treatment was not severe enough to

induce oxidative damage, especially during the reproductive season when resources are heavily allocated toward breeding.

To our knowledge, this is the first test of the impacts of acute stress exposure on sperm telomere dynamics in an animal. Further studies will be necessary to shape our understanding of the sensitivity of sperm telomere dynamics to organismal stress. Sperm are sensitive to damage, and may be sensitive to organismal stress on a shorter time scale than other cells due to their rapid generation and high turnover rate (Rodnan et al. 1956; Amir et al. 1973; Almbro et al. 2011; Losdat et al. 2011; Haussmann and Heidinger 2015). We found weak repeatability of sperm telomere length across a 14-day period, as compared to highly repeatable telomere length in red blood cells across a 6-month period. This suggests that sperm telomere length is susceptible to change over a relatively short period, and sperm telomere loss may occur in response to organismal stress that is severe enough to overcome any sperm-protective mechanisms.

Interestingly, in this study we found that sperm telomere length was positively correlated with blood telomere length within individuals, and that individuals' pre-treatment sperm and blood telomeres were not significantly different in length. In contrast, birds in our wild population have significantly longer sperm telomeres than blood telomeres while maintaining the positive correlation between the two (see Chapter 2). Sustained captivity is thought to be stressful for house sparrows, so this alone may be an indication that chronic stress can lead to sperm telomere degradation in birds; however, further investigation into this relationship is necessary (Martin et al. 2011).

References

Adelman JS, Bentley GE, Wingfield JC, Martin LB, Hau M. 2010. Population differences in fever and sickness behaviors in a wild passerine: a role for cytokines. J. Exp. Biol. 213:4099–4109.

Agilent Technologies. 2012. Introduction to quantitative PCR.

Almbro M, Dowling DK, Simmons LW. 2011. Effects of vitamin E and beta-carotene on sperm competitiveness. Ecol. Lett. 14:891–895.

Amir D, Braun-Eilon B, Schindler H. 1973. Passage and disappearance of labelled spermatozoa in the genital tract of the male Japanese quail in segregation or cohabitation. Ann. Biol. Anim. Biochim. Biophys. 13:321–328.

Ardia DR. 2005. Tree Swallows Trade off Immune Function and Reproductive Effort Differently Across Their Range. Ecology 86:2040–2046.

Asghar M, Bensch S, Tarka M, Hansson B, Hasselquist D. 2014. Maternal and genetic factors determine early life telomere length. Proc. R. Soc. B Biol. Sci. 282:20142263.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science. 347:436–438.

Atema E, Mulder E, Dugdale HL, Briga M, van Noordwijk AJ, Verhulst S. 2015. Heritability of telomere length in the Zebra Finch. J. Ornithol. 156:1113–1123.

Baird DM, Britt-Compton B, Rowson J, Amso NN, Gregory L, Kipling D. 2006. Telomere instability in the male germline. Hum. Mol. Genet. 15:45–51.

Beaulieu M, Reichert S, Le Maho Y, Ancel A, Criscuolo F. 2011. Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. Funct. Ecol. 25:577–585.

Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G. 2003. Assessing the Cost of Mounting an Immune Response. Am. Nat. 161:367–379.

Cawthon RM. 2002. Telomere measurement by quantitative PCR. Nucleic Acids Res. 30:47e–47.

Clark ME, Martin TE. 2007. Modeling tradeoffs in avian life history traits and consequences for population growth. Ecol. Modell. 209:110–120.

Costantini D. 2008. Oxidative stress in ecology and evolution: Lessons from avian studies. Ecol. Lett. 11:1238–1251.

Costantini D, Marasco V, Møller AP. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181:447–456.

Dantzer R, Kelley KW. 2007. Twenty years of research on cytokine-induced sickness behavior. Brain. Behav. Immun. 21:153–160.

Dunn AJ, Wang J. 1995. Cytokine effects on CNS biogenic amines. Neuroimmunomodulation 2:319–328.

Eisenberg DTA, Hayes MG, Kuzawa CW. 2012. Delayed paternal age of reproduction in humans is associated with longer telomeres across two generations of descendants. Proc. Natl. Acad. Sci. 109:10251–10256.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. 101:17312–17315.

Farner DS, Follett BK, King JR, Morton ML. 1966. A quantitiative examination of ovarian growth in the white-crowned sparrow. Biol. Bull. 130:67–75.

Ferlin A, Rampazzo E, Rocca MS, Keppel S, Frigo AC, De Rossi A, Foresta C. 2013. In young men sperm telomere length is related to sperm number and parental age. Hum. Reprod. 28:3370–3376.

Graakjaer J, Pascoe L, Der-Sarkissian H, Thomas G, Kolvraa S, Christensen K, Londoño-Vallejo JA. 2004. The relative lengths of individual telomeres are defined in the zygote and strictly maintained during life. Aging Cell 3:97–102.

Haussmann MF, Heidinger BJ. 2015. Telomere dynamics may link stress exposure and ageing across generations. Biol. Lett. 11:20150396.

Haussmann MF, Winkler DW, Huntington CE, Nisbet ICT, Vleck CM. 2007. Telomerase activity is maintained throughout the lifespan of long-lived birds. Exp. Gerontol. 42:610–618.

Heidinger BJ, Blount JD, Boner W, Griffiths K, Metcalfe NB, Monaghan P. 2012. Telomere length in early life predicts lifespan. Proc. Natl. Acad. Sci. 109:1743–1748.

Herborn KA, Heidinger BJ, Boner W, Noguera JC, Adam A, Daunt F, Monaghan P. 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. Proc. R. Soc. B Biol. Sci. 281:20133151.

Horn T, Robertson BC, Will M, Eason DK, Elliott GP, Gemmell NJ. 2011. Inheritance of telomere length in a bird. PLoS One 6:1–5.

Kelley KW, Bluthe R-M, Dantzer R, Zhou J-H, Shen W-H, Johnson RW, Broussard SR. 2003. Cytokine-induced sickness behavior Cytokine-induced sickness behavior. 1591:112–118.

Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, Cupples A, Hunkin JL, Gardner JP, Lu X, et al. 2008. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 4:1–9.

King JR, Farner DS. 1963. The relationship of fat deposition to Zugunruhe and migration. Condor 65:200–223.

King MO, Swanson DL. 2013. Activation of the immune system incurs energetic costs but has no effect on the thermogenic performance of house sparrows during acute cold challenge. J. Exp. Biol. 216:2097–2102.

Kucera AC, Heidinger BJ. 2018. Avian Semen Collection by Cloacal Massage and Isolation of DNA from Sperm. J. Vis. Exp.:e55324.

Lee KA, Martin LB, Wikelski MC. 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (Passer domesticus), than its less-invasive congener. Oecologia 145:244–251.

Lin M, Jones RC. 1992. Cell & Tissue Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail , Coturnix coturnix japonica. Cell & Tissue Res. 267:591–601.

Liu L, Bailey SM, Okuka M, Muñoz P, Li C, Zhou L, Wu C, Czerwiec E, Sandler L, Seyfang A, et al. 2007. Telomere lengthening early in development. Nat. Cell Biol. 9:1436–1441.

Losdat S, Richner H, Blount JD, Helfenstein F. 2011. Immune activation reduces sperm quality in the great tit. PLoS One 6.

Martin LB, Kidd L, Liebl AL, Coon CAC. 2011. Captivity induces hyper-inflammation in the house sparrow (Passer domesticus). J. Exp. Biol. 214:2579–2585.

Monaghan P. 2010. Telomeres and life histories: The long and the short of it. Ann. N. Y. Acad. Sci. 1206:130–142.

Monaghan P. 2014. Organismal stress, telomeres and life histories. J. Exp. Biol. 217:57–66.

Monaghan P, Haussmann MF. 2006. Do telomere dynamics link lifestyle and lifespan? Trends Ecol. Evol. 21:47–53.

Monaghan P, Heidinger BJ, D'Alba L, Evans NP, Spencer KA. 2012. For better or worse: reduced adult lifespan following early-life stress is transmitted to breeding partners. Proc. R. Soc. B Biol. Sci. 279:709–714.

Murton RK, Lofts B, Westwood NJ. 1970. Manipulation of photo-refractoriness in the house sparrow Passer domesticus by circadian light regimes. Gen. Comp. Endocrinol. 14:107–113.

Needham KB, Kucera AC, Heidinger BJ, Greives TJ. 2017. Repeated immune challenges affect testosterone but not sperm quality. J. Exp. Zool. Part A Ecol. Integr. Physiol. 327:398–406.

Njajou OT, Cawthon RM, Damcott CM, Wu S, Ott S, Garant MJ, Blackburn EH, Mitchell BD, Shuldiner AR, Hsueh W. 2007. Telomere length is paternally inherited and is associated with parental lifespan. Proc. Natl. Acad. Sci. U. S. A. 104:12135–9.

Nordfjäll K, Svenson U, Norrback K-F, Adolfsson R, Roos G. 2010. Large-scale parent-child comparison confirms a strong paternal influence on telomere length. Eur. J. Hum. Genet. 18:385–9.

Norris K, Evans MR. 2000. Ecological immunology : life history trade-offs and immune defense in birds. Behav. Ecol. 11:19–26.

Observatory USN. Data Services of the Astronomical Applications Department.

Owen-Ashley NT, Turner M, Hahn TP, Wingfield JC. 2006. Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (Zonotrichia leucophrys gambelii). Horm. Behav. 49:15–29.

Owen-Ashley NT, Wingfield JC. 2007. Acute phase responses of passerine birds: Characterization and seasonal variation. J. Ornithol. 148:583–591.

Reichert S, Rojas ER, Zahn S, Robin J-P, Criscuolo F, Massemin S. 2015. Maternal telomere length inheritance in the king penguin. Heredity (Edinb). 114:10–16.

de Reviers M. 1975. Sperm transport and survival in male birds. In: Hafez, E.S.E., Thibault CG, editor. The Biology of Spermatozoa. Basel, Switzerland: INSERM International Symposium, Nouzilly, S.: Karger. p. 10–16.

Richner H, Tripet F. 1999. Ectoparasitism and the Trade-Off between Current and Future Reproduction. Nord. Soc. Oikos 86:535–538.

Rodnan GP, Ebaugh FG, Spivey Fox MR. 1956. The life span of the red blood cell and the red blood cell volume in the chicken, pigeon and duck as estimated by the use of Na2Cr51O4. Blood 12:355–366.

Sköld-Chiriac S, Nord A, Nilsson J-Å, Hasselquist D. 2014. Physiological and Behavioral Responses to an Acute-Phase Response in Zebra Finches: Immediate and Short-Term Effects. Physiol. Biochem. Zool. 87:288–298.

Small TW, Sharp PJ, Deviche P. 2007. Environmental regulation of the reproductive system in a flexibly breeding Sonoran Desert bird, the Rufous-winged Sparrow, Aimophila carpalis. Horm. Behav. 51:483–495.

Turnbull AV, Lee S, Rivier C. 1998. Mechanisms of hypothalamic-pituitary-adrenal axis stimulation by immune signals in the adult rat. In: Annals of the New York Academy of Sciences. Vol. 840. p. 434–443.

Waites GMH. 2012. Temperature regulation and the testis. In: The Testis: Development, Anatomy, and Physiology. p. 241–279.

Wingfield JC, Sapolsky RM. 2003. Reproduction and Resistance to Stress: When and How. J. Neuroendocrinol. 15:711–724.

Wolfson A. 1952. The Cloacal Protuberance : A Means for Determining Breeding Condition in Live Male Passerines. Bird-Banding 23:159–165.

Zhao Z, Pan X, Liu L, Liu N. 2014. Telomere length maintenance, shortening, and lengthening. J. Cell. Physiol. 229:1323–1329.

CHAPTER 5: RELATIONSHIP BETWEEN TELOMERE LENGTHS IN SEVERAL TISSUES AND CHRONIC STRESS EXPERIENCE IN CAPTIVE MALE HOUSE SPARROWS

Abstract

Dysregulation of the HPA axis is a principle feature of chronic stress in animals, and is expected to accelerate telomere loss in birds, likely due to increased oxidative damage. Telomere length is most often measured in the blood, and little is known about telomere length across tissues in birds, particularly in relation to chronic stress exposure. The impact of chronic stress experience on sperm telomere length are of particular interest as direct transfer of gamete telomeres may be a mechanism of telomere length inheritance. We exposed captive male house sparrows to daily rotating stressors to induce chronic stress. We measured telomeres in five tissues; within individuals, blood telomeres were longest, organ telomeres were shortest, and sperm telomere length was intermediate. Fewer birds in the stress treatment produced semen, but telomere length did not differ between treatments in any tissue measured. Independent of treatment, birds with more depressed baseline CORT midway through captivity experienced greater blood telomere loss. The results of this study suggest that chronic stress is associated with telomere attrition in adult house sparrows.

Introduction

The stress response is an adaptive physiological mechanism that allows animals to survive acute threats and restore homeostasis (Sapolsky et al. 2000). Much of this response is orchestrated by the hypothalamic-pituitary-adrenal axis (HPA axis) and its rapid elevation of circulating corticosterone (CORT) in response to stressful stimuli (Wingfield et al. 1998; Sapolsky et al. 2000). In a typical avian acute stress response, elevation of CORT begins within

minutes of stressor exposure, and negative feedback at the hypothalamus and pituitary slowly reduce circulating glucocorticoids to baseline after reaching maximal levels within an hour (Romero and Romero 2002). Dysregulation of the HPA axis can occur when stressors are frequent or prolonged; when this occurs, the animal is said to be experiencing chronic stress (Rich and Romero 2005; Miller et al. 2007). This dysregulation of the HPA axis can manifest as either depressed or elevated CORT (baseline, stress-induced, and negative feedback), and may depend on stressor type and timing in relation to stressor onset (Breuner et al. 1999; Clinchy et al. 2004; Rich and Romero 2005; Miller et al. 2007; Dickens et al. 2009).

The consequences of chronic stress can persist long after exposure to the stressor, and can include accelerated aging and reduced longevity (Monaghan and Haussmann 2006; Miller et al. 2007; Monaghan et al. 2012; Monaghan 2014; Asghar et al. 2015; Hau et al. 2015). Although the causes of aging are not fully resolved, telomere dynamics (length and loss rate) is a potentially critical mechanism underlying longevity and therefore fitness (Monaghan 2010; Monaghan 2014).

Telomere loss occurs throughout life due to accumulating oxidative damage and normal DNA replication (Monaghan 2014; Zhao et al. 2014; Haussmann and Heidinger 2015). Stress exposure has been shown to accelerate telomere loss and reduce longevity (Epel et al. 2004; Monaghan 2014; Hau et al. 2015). The prevailing explanation for the link between chronic stress and accelerated telomere loss is based on the idea that HPA dysregulation usually involves elevated baseline CORT and slower negative feedback; greater exposure to glucocorticoids leads to increased oxidative damage (Costantini et al. 2011; Monaghan 2014). In cases where HPA dysregulation causes depressed CORT, it has been proposed that this could be protective of telomeres (Monaghan 2014). Alternatively, chronic stress with dampened CORT response is

associated with inflammation in house sparrows, and inflammation is also associated with increased oxidative damage; therefore, either type of HPA dysregulation may lead to accelerated telomere loss and reduced longevity (Federico et al. 2007; Martin et al. 2011).

Telomere length is most commonly measured in blood (leukocytes in mammals, erythrocytes in birds), as it is an easy tissue to sample noninvasively and has high cellular turnover (Haussmann et al. 2007). In adult animals, telomere loss rate is expected to be higher in highly proliferative tissues such as blood, gonads, and intestines, as compared to tissues with low cell division such as skeletal muscle and liver (Haussmann et al. 2007; Schmidt et al. 2016). Few studies have compared telomere length between tissues in birds, and in house sparrows it is unknown whether telomere length is correlated between tissues (Schmidt et al. 2016).

Sperm telomere length is of particular interest as direct inheritance of germ line telomeres is one hypothesized pathway of telomere inheritance (Haussmann and Heidinger 2015). Gonadal tissue is highly proliferative and also expresses telomerase throughout life in short-lived birds (Haussmann et al. 2007). Sperm are highly susceptible to oxidative damage, but upregulated telomerase expression in the gonad may protect gamete telomeres against damage caused by increased oxidative stress, such as during HPA dysregulation in chronic stress (Almbro et al. 2011; Losdat et al. 2011; Haussmann and Heidinger 2015).

We exposed captive adult male house sparrows to daily rotating stressors to address the following questions: 1) Is telomere length between tissues correlated within individuals? 2) Does chronic stress lead to blood telomere loss or tissue-specific differences in telomere length? 3) Does chronic stress during the non-reproductive season impact sperm telomere length after gonadal recrudescence? Based on the extremely limited number of previous studies, we predicted low correlation between telomere length in blood and non-proliferative tissues (liver,

skeletal muscle, heart), and a positive correlation between telomere length in blood and sperm (Schmidt et al. 2016; Chapters 3 and 4). Additionally, we expected chronic stress to have a greater impact on telomere length in proliferative tissues as compared to non-proliferative tissues. However, it is unclear to what degree sperm telomeres could be buffered against the consequences of stress by mechanisms such as high telomerase expression and selective destruction of low-quality sperm (Haussmann et al. 2007; Kimura et al. 2008; Eisenberg et al. 2012).

Methods

Study subjects

Mist nets were used to capture 30 adult male house sparrows around Fargo, ND in early 2017. Males were individually housed in wire cages (23" wide x 16" deep x 16" tall) on racks in two rooms at North Dakota State University. All cages were visually and acoustically open to the other birds in the room, and the male cage racks were positioned about 3 feet apart with a centrally located cage stand housing 4 females, which were visible to all males. Food (Kaytee supreme mixed seed for finches), water, and cuttlebones were provided *ad libitium*, and supplemental food (broccoli, baby spinach, peas and carrots, or Quicko Classic Egg Food) was provided once per week.

Following capture, the bird rooms were kept on a short-day light cycle of 8L:16D to keep the birds in non-reproductive condition (gonadally regressed) throughout the first treatment period. At the end of the chronic stress phase of captivity, the light cycle was changed to a long-day cycle of 16L:8D to photostimulate the birds and trigger gonadal recrudescence (King and Farner 1963; Small et al. 2007). During the breeding season in Fargo, ND, free-living sparrows experience a maximal day length of 15 hours and 52 minutes, closely matching the experimental

long-day light cycle (U.S. Naval Observatory). The room temperature was maintained between 21-23 °C for the duration of captivity. Animal care and experimental procedures were approved by the IACUC at North Dakota State University (#A17035).

Chronic stress treatment

Birds were randomly assigned to either experimental or control treatment upon capture (n = 15 males per treatment). One week after the last bird was captured, the chronic stress protocol was initiated (Figure 12). During the chronic stress phase, the birds in the stress room were exposed to one of five stressors daily, applied in a randomly generated order and at a random time each day between the hours of 10am and 10pm. Each stressor was applied for 30 minutes. At the same time as the initiation of the stressor each day, the control room received a non-treatment protocol of opening and immediately closing the door without entry. The five experimental stressors were selected to mimic stressful events that house sparrows might be exposed to in the wild.

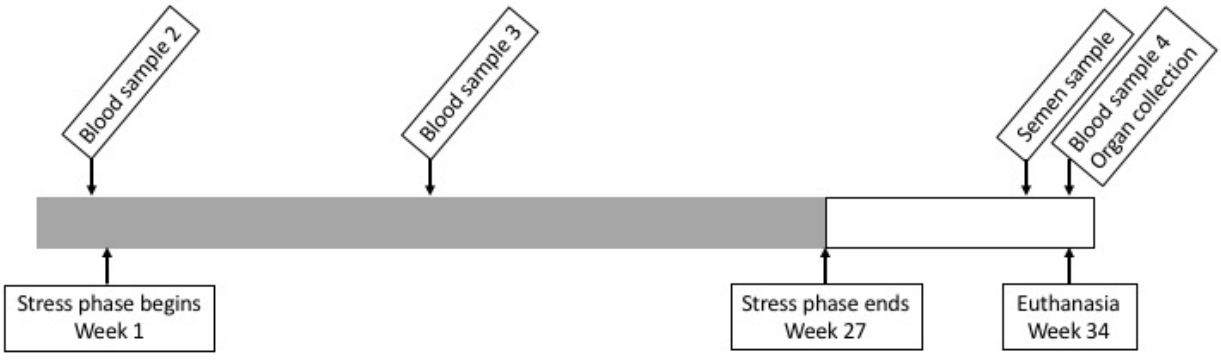

Figure 12. Timeline of chronic stress experiment in adult male house sparrows. The shaded box represents short-day light cycle and the white box represents long-day light cycle.

The five stressors were a live avian predator (common kestrel, *Falco tinnunculus*), a live feline predator (domestic cat, *Felis catus*), a mounted avian predator (merlin, *Falco columbarius*), loud radio (to represent anthropogenic acoustic disturbance), and rattling and

shaking of the cage racks (to represent physical disturbances such as high winds or movement/rattling of nest cavity). The live predators were presented twice as often as the other three stressors; on average, birds experienced each live predator twice per week, and each of the other stressors once per week. During each live predator presentation, the predator was allowed to move around the room and get very close to the cages. The cat roamed the room and vocalized, climbed the cage racks, and stared at the caged sparrows from close distances. The kestrel flew across the room, wing flared at the sparrows, stared at the caged sparrows from close distances, and ate whole dead mice in view of the sparrows. The chronic stress phase lasted 27 weeks, after which both rooms received maintenance care only as they adjusted to the long-day light cycle and came into reproductive condition.

Blood and semen sampling

Blood was collected several times for telomere measurement and quantification of plasma CORT. At each blood sampling event approximately 75 μL of whole blood was collected via brachial venipuncture into heparinized micro-hematocrit tubes. The blood was stored on ice for up to 2 hours, centrifuged, separated into plasma and red blood cell fractions, and frozen at -80 °C until hormone measurement. All baseline samples were collected less than 3 minutes after opening the door to the bird room.

An initial blood sample was collected from each individual upon capture (sample 1). Prior to the initiation of the chronic stress phase of captivity, after all birds had been in captivity at least one week, a blood sample was collected from each male to serve as baseline for pre-treatment telomere length and CORT (sample 2; Figure 12). Two further samples were collected, 7 weeks into the chronic stress phase (sample 3) and at euthanasia (after semen collection;

sample 4). Telomere lengths were compared between samples 2 and 4 to measure change in telomere length across the experiment.

Semen was collected by cloacal massage after the birds had been on long-day light cycle for 6 weeks (Wolfson 1952; Kucera and Heidinger 2018). At this time, all birds in the control treatment had recrudesced gonads and were producing semen. Semen samples were collected into heparinized micro-hematocrit tubes and immediately diluted into 20 μL of 1x PBS. These samples were stored on ice for up to an hour, then stored at -80 °C until DNA extraction. Due to euthanasia of 4 birds during the experiment for health reasons, at semen collection each treatment contained 13 birds. Of these, 100% of the control group produced semen while 70% of the stress birds produced semen.

Organ collection

All birds were euthanized a week after semen collection (34 weeks after onset of the chronic stress phase, 7 weeks after the end of chronic stress and start of the long-day light cycle). The following tissues were dissected and immediately frozen on dry ice: left pectoralis muscle, heart, and right lobe of liver.

Measurement of telomeres

DNA was extracted from red blood cells and organs using commercially available extraction kits (Macherey-Nagel NucleoSpin Blood #740951, Macherey-Nagel NucleoSpin Tissue #740952) and following manufacturer protocol. DNA was extracted from whole diluted semen samples using commercially available extraction kits with modifications, following Kucera and Heidinger (2018). After extraction, DNA concentration and purity were assessed with a Nanodrop 8000 spectrophotometer (Thermo Scientific).

Relative telomere length was measured using quantitative PCR on a Strategene Mx3000P as described in Cawthon (2002) and modified for use in house sparrows. The relative telomere length of samples was calculated as the ratio (T/S) between telomere repeat copy number (T) and single copy control gene number (S) relative to the reference sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single copy control gene. The telomere forward and reverse primers used were: Tel 1B (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′), Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′). Zebra finch GAPDH primers were used to amplify the control gene: GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3'), GAPDH-R (5'CCATCAGCAGCAGCCTTCA-3'). The telomere and GAPDH reactions were run on separate plates, and the number of PCR cycles (C_t) required for the products to accumulate enough fluorescence to cross a threshold was determined each time. Samples with relatively low C_t values had longer telomeres than those with relatively high C_t values. All samples were run in triplicate, and average values were used to determine the T/S ratio using the following formula: $2^{\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{telo} - C_t^{GAPDH})$ reference - $(C_t^{telo} - C_t^{GAPDH})$ (Agilent Technologies 2012).

The efficiency of each reaction was measured by including on each plate a standard curve produced by serial dilution of a reference sample. This sample was a pool of DNA from several female house sparrows collected in 2014. In all cases, plate efficiencies were within the accepted range of 100 ± 15%, and samples fell within the bounds of the standard curve. Intra-plate variation of Ct for GAPDH and telomere plates were 0.51% and 0.71% respectively, and inter-plate variation of ΔCt was 6.03%.

Hormone quantification

A commercially available CORT enzyme immunoassay kit (Enzo Life Sciences ADI-901-097) was used for CORT analysis following a protocol previously validated in house sparrows (Kuhlman and Martin 2010; Martin et al. 2011). Briefly, 5 μL of 10% steroid displacement buffer was added to 5 μL of each plasma sample, 240 μL of assay buffer was added, and the sample mixture was aliquoted in duplicate to the assay plate. A standard curve ranging from 32 – 200,000 pg/ml was aliquoted in triplicate. All wells were incubated with conjugated CORT and antibody on a shaker, wells were washed, and substrate was added before a second incubation. Stop solution was added, and the plate was read at 405 nm (corrected at 580 nm per manufacturer's recommendation). Inter- and intra-assay variation were 8.07% and 2.43% respectively.

Statistical analysis

To ensure normality, all T/S ratios and CORT values were natural log-transformed. We used independent samples t-tests to determine whether telomere length or change in mass differed between treatments. We used linear mixed models to test for relationships between telomere length and loss, mass, and baseline CORT. Pearson correlations were used to assess correlations in telomere length between tissues.

Mixed model for baseline CORT between pre-experiment and mid-experiment samples: CORT was the dependent variable, with treatment and time point as fixed effects, and bird as a random effect to control for repeated measures. Mixed model for relationship between blood telomere length and mass: telomere length at sample 4 was the dependent variable, with change in mass and telomere length at sample 2 as fixed effects, and plate and bird as random effects. Mixed model for relationship between sperm telomere length, change in blood telomere length,

and change in mass: sperm telomere length was the dependent variable, with change in blood telomere length and change in mass as fixed effects, and plate and bird as random effects. Mixed model for relationship between change in blood telomere length and baseline CORT: change in blood telomere length was the dependent variable, with mid-experiment baseline CORT as a fixed effect, and pre-experiment baseline CORT and plate as random effects. Mixed model for relationship between sperm telomere length and baseline CORT: sperm telomere length was the dependent variable, with mid-experiment baseline CORT as a fixed effect, and pre-experiment baseline CORT and plate as random effects. Mixed model for telomere length by tissue type: telomere length was the dependent variable, with tissue and mid-experiment baseline CORT as fixed effects, and bird as a random effect.

Results

Baseline CORT was lower in the mid-experiment sample than prior to beginning the chronic stress protocol ($F_{1, 22.66} = 8.86$, $p = .007$), but was not influenced by treatment ($F_{1, 23.53} = .194$, $p = .664$). Birds in the stress treatment lost more mass than birds in the control treatment ($p < .001$). Change in telomere length between samples 2 and 4, sperm telomere length, heart telomere length, liver telomere length, and pectoralis telomere length did not differ between treatments. Within individuals, blood and sperm telomere length were positively correlated ($r = .53$, $p = .016$), blood and heart telomere length were positively correlated ($r = .47$, $p = .026$), and no other tissues were correlated (Table 2).

Table 2. Correlations between telomere lengths in blood, sperm, heart, liver, and pectoralis muscle in adult male house sparrows in captivity.

Tissue	Sperm	Liver	Heart	Pectoralis
Blood	**r = 0.53** **p = .016**	r = 0.34 p = .11	**r = 0.47** **p = .026**	r = -0.38 p = .079
Sperm		r = 0.03 p = .91	r = 0.38 p = .11	r = 0.04 p = .88
Liver			r = -0.13 p = .59	r = .18 p = .44
Heart				r = -0.29 p = .21

P-values below 0.05 are indicated in bold.

Terminal blood telomere length (sample 4) was not related to change in mass, but was significantly related to an individual's telomere length at sample 2 ($F_{1,22.99} = 8.89$, $p = .007$), such that birds with longer telomeres at the start of the experiment also had longer telomeres at the end. Sperm telomere length was not related to an individual's change in blood telomere length or change in mass. Change in blood telomere length was related to mid-experiment baseline CORT ($F_{1,14.25} = 8.87$, $p = .01$) such that birds with lower baseline CORT experienced greater telomere loss. There was no relationship between sperm telomere length and baseline CORT. Within individuals, telomere length was significantly shorter in heart, pectoralis, and liver as compared to blood, and sperm telomere length was intermediate to blood and the other three tissues ($F_{5,94.37} = 58.99$, $p < .0001$; Figure 13, Blood = A, sperm = B, liver heart pec = C), but telomere length between tissues was not related to baseline CORT ($F_{1,35.36} = .18$, $p = .67$).

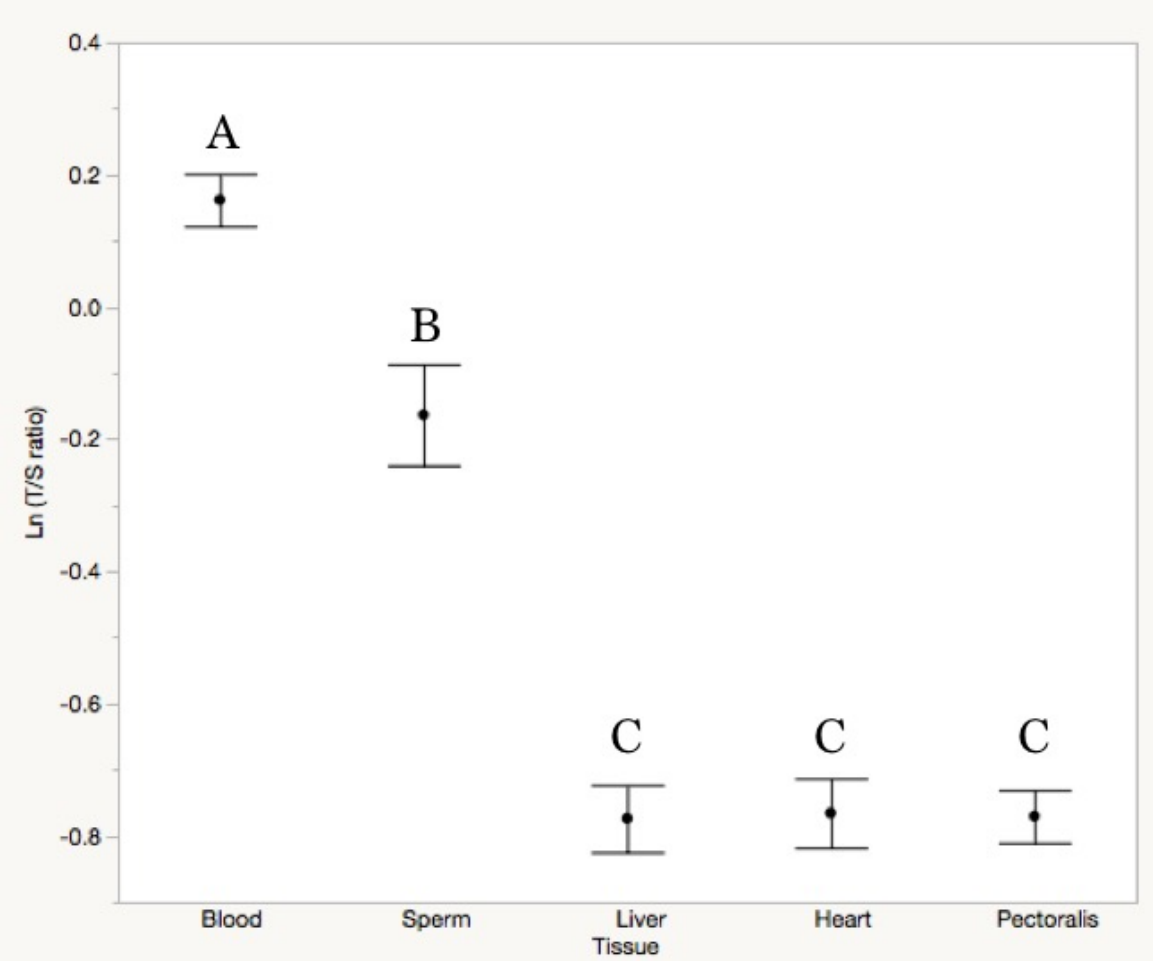

Figure 13. Telomere length (± SEM, reported as Ln[T/S ratio] measured by qPCR) in blood, sperm, heart, liver, and pectoralis muscle of adult male house sparrows in captivity. Letters indicate significant differences between tissues.

Discussion

Dysregulation of the HPA axis is a principle feature of chronic stress in animals, and is expected to accelerate telomere loss in birds, likely due to increased oxidative damage. Telomere length is most often measured in the blood, and little is known about telomere length across tissues in birds, particularly in relation to chronic stress exposure. We are especially interested in the impacts of chronic stress experience on sperm telomere length, as direct transfer of gamete telomeres may be a mechanism of telomere length inheritance.

We exposed captive male house sparrows to daily rotating stressors in an attempt to induce chronic stress. Birds in the stress treatment lost significantly more mass than those in the control treatment, but all birds showed evidence of HPA dysregulation independent of treatment (depressed baseline CORT midway through captivity). These changes in mass and baseline CORT suggest that all birds were experiencing chronic stress in response to captivity, but the stress group may have been somewhat more stressed than the control group (Rich and Romero 2005). In support of this, only 70% of birds in the stress treatment produced semen, compared to 100% in the control treatment, suggesting a negative impact of chronic stress on reproduction. Alternatively, both groups were experiencing chronic stress in response to captivity, but birds in the stress treatment plastically adjusted their body mass in response to perceived high predation risk (frequent live predator presence; Lima 1986; Rogers 1987; Gosler et al. 1995). House sparrows seem to habituate poorly to captivity, and captivity alone is thought to be stressful for this species (Kuhlman and Martin 2010; Martin et al. 2011).

Telomere length did not differ between treatments in any tissue measured. Independent of treatment, birds with more depressed baseline CORT midway through captivity also experienced greater telomere loss. This supports the idea that the rotating stressor treatment did not cause an additional chronic stress load over the stress of captivity, but suggests that individual variation in stress sensitivity may be associated with individual outcomes in telomere dynamics. On average, blood telomeres did not significantly shorten across the experiment (8 months).

In a similar experiment, Hau et al. (2015) exposed adult Eurasian blackbirds (*Turdus merula*) to repeated stressors and found accelerated blood telomere loss and increased oxidative damaged compared to control birds. These blackbirds were hand-raised in captivity and exposed to 4 lipopolysaccharide (LPS) injections and 120 rotating stressors (4 per day across 3 sets of 10

days) across the span of a year (Hau et al. 2015). Our stressor protocol was less intense but more consistent, with one daily rotating stressor occurring for 190 days, in comparison to Hau et al. (2015) with more intense but sporadic rotating stressors. The key differences between these two studies are in species and captivity status. While the blackbirds were hand-raised adults, our house sparrows were captured as adults between one week and several weeks prior to the start of the experiment. The finding of consistently elevated oxidative damaged in chronically stressed blackbirds and accelerated telomere attrition suggests oxidative damage is involved in mediating telomere loss in adult birds (Hau et al. 2015). While our birds were likely chronically stressed by captivity, we did not measure oxidative damage or antioxidant capacity, which may have played a role in individual variation in telomere loss.

Within individuals, we found that blood telomere length was significantly longer than sperm telomere length, and both blood and sperm telomeres were longer than heart, liver, and pectoralis telomeres. Blood telomere length was positively correlated with both sperm and heart telomere length. In contrast, Reichert et al. (2013) found that pectoralis telomeres were significantly longer than liver telomeres, but reported no other differences between the same somatic tissues measured here. In those finches, several positive correlations were found between tissues. Further, Schmidt and Heidinger (2016) found no significant differences in telomere length between the same somatic tissues, or correlations between tissue-specific telomere length, in juvenile Franklin's gulls. While the discrepancies between these three results are somewhat surprising, very little is currently known about how telomere lengths in different tissues are correlated within individuals or how this may differ between species.

Telomeres in pectoralis muscle and liver tissue may be at greater risk of attrition during chronic stress compared to other tissues. In house sparrows, glucocorticoid receptor expression

increases in the pectoralis following chronic stress exposure, while this increase is not found in another region of skeletal muscle (Lattin and Romero 2014). Glucocorticoid receptors bind stress-induced CORT and stimulate proteolysis, which is catabolic muscle wasting that provides free amino acids for gluconeogenesis (Lattin and Romero 2014). Proteolysis is associated with elevated oxidative stress; therefore, chronic stress may lead to elevated oxidative damage and telomere attrition particularly in pectoralis muscle in birds (Chalmeh et al. 2015). Liver tissue may also be subject to elevated oxidative damage in response to stress as glycogenolysis occurs primarily in the liver as an additional catabolic process to provide additional glucose (Siegel 1980). Additionally, a more tenuous link may exist for heart tissue: elevated heart rate is associated with elevated oxidative stress, and enhanced heart rate and cardiovascular tone are features of HPA activation (Sapolsky et al. 2000; Custodis et al. 2008; Cyr et al. 2009).

The results of this study suggest that chronic stress impacts blood telomere length but not sperm telomere length. Individuals with the strongest depression of baseline CORT experienced greater blood telomere loss, but sperm telomere length was not related to baseline CORT. Sperm telomeres may be protected from the effects of stress by suppression of testicular recrudescence, as only 70% of birds in the chronic stress treatment produced semen after six weeks of photostimulation. House sparrows may not be the ideal species for studies of this nature, since they are known to react negatively to captivity, and may also be more resistant to the consequences of stress than other passerines (Helfenstein et al. 2010; Martin et al. 2011; King and Swanson 2013).

References

Agilent Technologies. 2012. Introduction to quantitative PCR.

Almbro M, Dowling DK, Simmons LW. 2011. Effects of vitamin E and beta-carotene on sperm competitiveness. Ecol. Lett. 14:891–895.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science. 347:436–438.

Breuner CW, Wingfield JC, Romero LM. 1999. Diel rhythms of basal and stress-induced cort in wild white-crowned sparrow. J. Exp. Zool. 284:334–342.

Cawthon RM. 2002. Telomere measurement by quantitative PCR. Nucleic Acids Res. 30:47.

Chalmeh AA, Nazifi S, Pourjafar M, Zarei MR. 2015. Oxidative stress and protein catabolism following dexametha- sone and isoflupredone administration in Holstein calves. Iran. J. Vet. Med. 9:233–239.

Clinchy M, Zanette L, Boonstra R, Wingfield JC, Smith JNM. 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proc. R. Soc. B Biol. Sci. 271:2473–2479.

Costantini D, Marasco V, Møller AP. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181:447–456.

Custodis F, Baumhäkel M, Schlimmer N, List F, Gensch C, Böhm M, Laufs U. 2008. Heart rate reduction by ivabradine reduces oxidative stress, improves endothelial function, and prevents atherosclerosis in apolipoprotein E-deficient mice. Circulation 117:2377–2387.

Cyr NE, Dickens MJ, Romero LM. 2009. Heart rate and heart-rate variability responses to acute and chronic stress in a wild-caught passerine birds. Physiol. Biochem. Zool. 82:332–344.

Dickens M, Romero LM, Cyr NE, Dunn IC, Meddle SL. 2009. Chronic stress alters glucocorticoid receptor and mineralocorticoid receptor mRNA expression in the European starling (Sturnus vulgaris) brain. J. Neuroendocrinol. 21:832–840.

Eisenberg DTA, Hayes MG, Kuzawa CW. 2012. Delayed paternal age of reproduction in humans is associated with longer telomeres across two generations of descendants. Proc. Natl. Acad. Sci. 109:10251–10256.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. 101:17312–17315.

Federico A, Morgillo F, Tuccillo C, Ciardiello F, Loguercio C. 2007. Chronic inflammation and oxidative stress in human carcinogenesis. Int. J. Cancer 121:2381–2386.

Gosler AG, Greenwood JJD, Perrins C. 1995. Predation risk and the cost of being fat. Nature 377:621–623.

Hau M, Haussmann MF, Greives TJ, Matlack C, Costantini D, Quetting M, Adelman JS, Miranda AC, Partecke J. 2015. Repeated stressors in adulthood increase the rate of biological ageing. Front. Zool. 12:1–10.

Haussmann MF, Heidinger BJ. 2015. Telomere dynamics may link stress exposure and ageing across generations. Biol. Lett. 11:20150396.

Haussmann MF, Winkler DW, Huntington CE, Nisbet ICT, Vleck CM. 2007. Telomerase activity is maintained throughout the lifespan of long-lived birds. Exp. Gerontol. 42:610–618.

Helfenstein F, Podevin M, Richner H. 2010. Sperm morphology, swimming velocity, and longevity in the house sparrow Passer domesticus. Behav. Ecol. Sociobiol. 64:557–565.

Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, Cupples A, Hunkin JL, Gardner JP, Lu X, et al. 2008. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 4:1–9.

King MO, Swanson DL. 2013. Activation of the immune system incurs energetic costs but has no effect on the thermogenic performance of house sparrows during acute cold challenge. J. Exp. Biol. 216:2097–2102.

Kucera AC, Heidinger BJ. 2018. Avian Semen Collection by Cloacal Massage and Isolation of DNA from Sperm. J. Vis. Exp.:e55324.

Kuhlman JR, Martin LB. 2010. Captivity affects immune redistribution to skin in a wild bird. Funct. Ecol. 24:830–837.

Lattin CR, Romero LM. 2014. Chronic stress alters concentrations of corticosterone receptors in a tissue-specific manner in wild house sparrows (Passer domesticus). J. Exp. Biol. 217:2601–2608.

Lima SL. 1986. Predation risk and unpredictable feeding conditions: determinants of body mass in birds. Ecology 67:377–385.

Losdat S, Richner H, Blount JD, Helfenstein F. 2011. Immune activation reduces sperm quality in the great tit. PLoS One 6.

Martin LB, Kidd L, Liebl AL, Coon CAC. 2011. Captivity induces hyper-inflammation in the house sparrow (Passer domesticus). J. Exp. Biol. 214:2579–2585.

Miller GE, Chen E, Zhou ES. 2007. If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychol. Bull. 133:25–45.

Monaghan P. 2010. Telomeres and life histories: The long and the short of it. Ann. N. Y. Acad. Sci. 1206:130–142.

Monaghan P. 2014. Organismal stress, telomeres and life histories. J. Exp. Biol. 217:57–66.

Monaghan P, Haussmann MF. 2006. Do telomere dynamics link lifestyle and lifespan? Trends Ecol. Evol. 21:47–53.

Monaghan P, Heidinger BJ, D'Alba L, Evans NP, Spencer KA. 2012. For better or worse: reduced adult lifespan following early-life stress is transmitted to breeding partners. Proc. R. Soc. B Biol. Sci. 279:709–714.

Observatory USN. U.S. Naval Observatory.

Reichert S, Criscuolo F, Verinaud E, Zahn S, Massemin S. 2013. Telomere length correlations among somatic tissues in adult zebra finches. PLoS One 8:1–7.

Rich EL, Romero LM. 2005. Exposure to chronic stress downregulates corticosterone responses to acute stressors. AJP Regul. Integr. Comp. Physiol. 288:R1628–R1636.

Rogers CM. 1987. Predation Risk and Fasting Capacity: Do Wintering Birds Maintain Optimal Body Mass? Ecology 68:1051–1061.

Romero LM, Romero RC. 2002. Corticosterone Responses in Wild Birds : The Importance of Rapid Initial Sampling. Condor 104:129–135.

Sapolsky RM, Romero LM, Munck AU. 2000. How Do Glucocorticoids Influence Stress Responses ? Endocr. Rev. 21:55–89.

Schmidt JE, Sirman AE, Kittilson JD, Clark ME, Reed WL, Heidinger BJ. 2016. Telomere correlations during early life in a long-lived seabird. Exp. Gerontol. 85:28–32.

Siegel HS. 1980. Physiological Stress in Birds. Bioscience 30:529–534.

Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M, Richardson RD. 1998. Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage." 38:191–206.

Wolfson A. 1952. The Cloacal Protuberance : A Means for Determining Breeding Condition in Live Male Passerines. Bird-Banding 23:159–165.

Zhao Z, Pan X, Liu L, Liu N. 2014. Telomere length maintenance, shortening, and lengthening. J. Cell. Physiol. 229:1323–1329.

CHAPTER 6: SCARY CATS AND NERVOUS BIRDS: CORTICOSTERONE RESPONSES TO LIVE PREDATOR PRESENCE IN CAPTIVE HOUSE SPARROWS

Abstract

The capture-restraint protocol is frequently used as a simple field measure to approximate responsiveness to stressors in birds. As a standardized method, capture-restraint is very useful for comparing between species, between studies, and for repeated measures of individuals across time. However, few studies have considered the relationship between individual response to the capture-restraint protocol and an ecologically-relevant stressor. How birds respond to different stressors or different types of stressors has been the subject of very little attention. We measured CORT response to three different stressor types (capture-restraint and two live predators). Here, we show that individual CORT response to one predator is consistent across time, but response varies by stressor type. This suggests that individual responses to stressors is context-dependent.

Introduction

Activation of the stress response is a critical mechanism by which vertebrates are able to survive acute challenges. A major part of this response involves the hypothalamic-pituitary-adrenal axis (HPA axis), activation of which causes a rapid elevation of circulating glucocorticoid hormones from the adrenal glands, especially corticosterone (CORT) in birds (Wingfield et al. 1998; Sapolsky et al. 2000). At baseline levels, CORT is involved in regulating energy metabolism, feeding behavior, locomotion, and reproduction (Sapolsky et al. 2000; Wingfield and Sapolsky 2003; Landys et al. 2006). Stress-induced levels of CORT lead to behavioral and metabolic changes that favor immediate survival of the animal, but potentially at a cost to reproduction or future survival (Wingfield 1994; Sapolsky et al. 2000; Scheuerlein et al. 2001; Wingfield and Sapolsky 2003; Landys et al. 2006; Monaghan 2014; Marasco et al. 2015).

In birds, elevation of circulating CORT begins within minutes of stressor exposure, and typically reaches a maximal level within an hour, after which negative feedback at the hypothalamus and pituitary slowly reduce circulating glucocorticoids to baseline (Romero and Romero 2002).

A standardized capture-restraint protocol is frequently used to measure baseline and maximal CORT in birds (Wingfield et al. 1982; Wingfield et al. 1992; Lendvai et al. 2007). This handling and restraint protocol is a simple procedure that has been widely used to approximate individual sensitivity to stress: a bird is captured, a baseline sample is taken in under 3 minutes, and the bird is restrained in a fabric bag for a standardized length of time until the maximal blood sample is collected. While context-dependent stress responsiveness is a topic of broad interest, few studies have considered how individual stress responsiveness varies with stressor type in birds (Silverin 1998; Cockrem and Silverin 2002a; Butler et al. 2009; Canoine et al. 2013). In particular, an open question remains as to how an individual's response to the capture-restraint protocol relates to their response to an ecologically-relevant stressor such as predator exposure.

Does knowing how a bird responds to stress in one context tell you about how it responds in other contexts? To approach this question, we exposed captive house sparrows to two different live predators as well as the standardized capture-restraint protocol, and compared their CORT responses to each stressor. The live predators were a domestic cat (*Felis catus*) and a common kestrel (*Falco tinnunculus*), both of which are known predators of house sparrows. The domestic cat is a significant predator of small birds in general, and of house sparrows specifically (Churcher and Lawton 1987; Woods et al. 2003; Bonnington et al. 2013). Common kestrels may kill fewer house sparrows than free-ranging cats, but a large portion of their diet is comprised of house sparrows where they co-occur, particularly in urban environments (Yalden 1980; Kübler et al. 2005; Gentle and Kettel 2013). We also compared the birds' response to the live cat over time

to determine a) whether they habituate to repeated predator exposure and b) the consistency of individual responses to a given stressor.

Methods

Study subjects

Mist nets were used to capture 15 adult male house sparrows around Fargo, ND in early 2017. Birds were individually housed in wire cages (23" wide x 16" deep x 16" tall) in one room at North Dakota State University for up to 12 months. The cages were visually open to the rest of the room, and cage racks were placed about 3 feet apart with a centrally located cage stand housing 4 females, which were visible to all males. Food (Kaytee Supreme mixed seed for finches), water, and cuttlebones were provided *ad libitum*, and supplemental food (broccoli, baby spinach, peas and carrots, or Quicko Classic Egg Food) was provided once per week.

Following capture, birds were kept on a short-day light cycle of 8L:16D throughout the experiment. The room temperature was maintained between 21-23 °C for the duration of captivity. Animal care and experimental procedures were approved by the IACUC at North Dakota State University (#A17035).

Stress exposure

As part of another experiment testing the effects of chronic stress on telomere dynamics (see Chapter 5), the birds were exposed to daily rotating stressors for 27 weeks. During this chronic stress phase of captivity, each day at a randomized time during the light hours, the birds were exposed to one of five randomized rotating stressors for 30 minutes: live domestic cat, live common kestrel, taxidermy merlin (*Falco columbarius*), cage shaking/banging, and loud rock music. The live predators were presented twice as often as the other three stressors; on average, birds experienced each live predator twice per week, and each of the other stressors once per

week. During each live predator presentation, the predator was allowed to move around the room and get very close to the cages. The cat roamed the room and vocalized, climbed the cage racks, and stared at the caged sparrows from close distances. The kestrel flew across the room, wing flared at the sparrows, stared at the caged sparrows from close distances, and ate whole dead mice in view of the sparrows.

Blood sampling

Blood was collected for quantification of plasma CORT six times. At each collection, approximately 75 μL of whole blood was collected from the brachial vein into heparinized micro-hematocrit tubes. Blood samples were stored on ice for up to 2 hours, centrifuged, separated into plasma and red blood cell fractions, and frozen at -80 °C until hormone measurement. All baseline samples were collected less than 3 minutes after opening the door to the bird room, and maximal or stress-induced samples were collected after 30 minutes of stress exposure, as previous studies have shown that 30 minutes of restraint elicits maximal circulating CORT in house sparrows (Breuner and Orchinik 2001; Lindstrom et al. 2005).

Three days prior to the beginning of the chronic stress phase of captivity (and when all birds had been in captivity at least 1 week), a pre-experiment baseline sample was collected from each bird (Figure 14). Two stress-induced samples were collected after 30 minutes of cat exposure: the first time they were exposed to the cat, and 35 days later (approximately the 10th time they were exposed to the cat). One baseline and one maximal handling-induced sample were collected on day 156 of daily stressor exposure. One stress-induced sample was collected after 30 minutes of kestrel exposure on day 170 of stressors. Some samples were missing at certain time points, and the sample sizes by sampling event were as follows: pre-experiment baseline (13), cat 1 (15), cat 2 (15), mid-experiment baseline (13), capture-restraint (14), falcon

(13). While the rotating stressors were presented at variable times during the day throughout the chronic stress phase of captivity, on sampling days the stressor and sampling were always done at the same time of day.

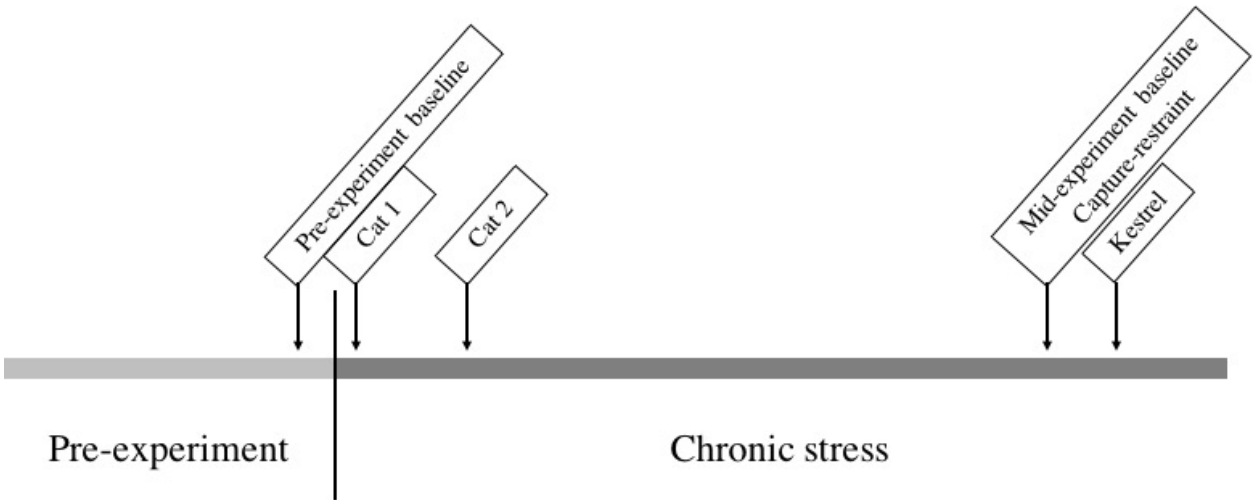

Figure 14. Timeline of CORT sampling events from captive male house sparrows exposed to multiple stressors. A total of six samples were collected across 180 days. During the chronic stress phase of captivity, birds were exposed to a rotating stressor for 30 minutes each day.

Hormone quantification

A commercially available CORT enzyme immunoassay kit (Enzo Life Sciences ADI-901-097) was used for CORT analysis following a protocol previously validated in house sparrows (Kuhlman and Martin 2010; Martin et al. 2011). Briefly, 5 μL of 10% steroid displacement buffer was added to 5 μL of each plasma sample, 240 μL of assay buffer was added, and the sample mixture was aliquoted in duplicate to the assay plate. A standard curve ranging from 32 – 200,000 pg/ml was aliquoted in triplicate. All wells were incubated with conjugated CORT and antibody on a shaker, wells were washed, and substrate was added before a second incubation. Stop solution was added, and the plate was read at 405 nm (corrected at 580 nm per manufacturer's recommendation). Inter- and intra-assay variation were 8.07% and 2.43% respectively.

Statistical analysis

To determine whether individual differences in CORT response were maintained across stressor types, we used a linear mixed effects model. The dependent variable was ln(CORT[pg]), natural log transformed to achieve normality. Stressor type was included as a fixed effect, and individual was included as random effect. Mass was not included as a random effect because we did not have a mass measurement for every sampling point, and CORT was not correlated with mass. A least square difference test (LSD) was used to determine which stressor types differed in CORT response. To determine how individual responses were related between each stressor type, we used linear correlations of ln(CORT[pg]). We used a t-test to test whether baseline CORT differed between pre-experiment and mid-experiment collections. To estimate repeatability of CORT response across stressors, we used rptR for R 3.5.0 (Schielzeth and Nakagawa 2011). All other analyses were performed in SPSS 24 (IBM SPSS).

Results

There was a significant effect of stressor type on CORT response when controlling for individual ($F_{3, 39.64} = 26.73$, $p < .0001$; Figure 15). Maximal CORT was lowest in response to the kestrel ($p < .001$ compared to each other stressor), and did not differ between cat 1, cat 2, and capture-restraint. Pre-experiment baseline CORT was positively correlated with the first cat exposure ($R^2 = .63$, $p = .021$). Cat 1 was positively correlated with both cat 2 ($R^2 = .62$, $p = .014$; Figure15) and falcon ($R^2 = .66$, $p = .021$), and cat 2 was also positively correlated with capture-restraint ($R^2 = .61$, $p = .021$; also see Table 3). Individual maximal CORT was repeatable across all four stressor samples ($R = .232$, $p = .038$; Figure 16). Baseline CORT was lower at the mid-experiment sample than the pre-experiment sample ($p = .049$).

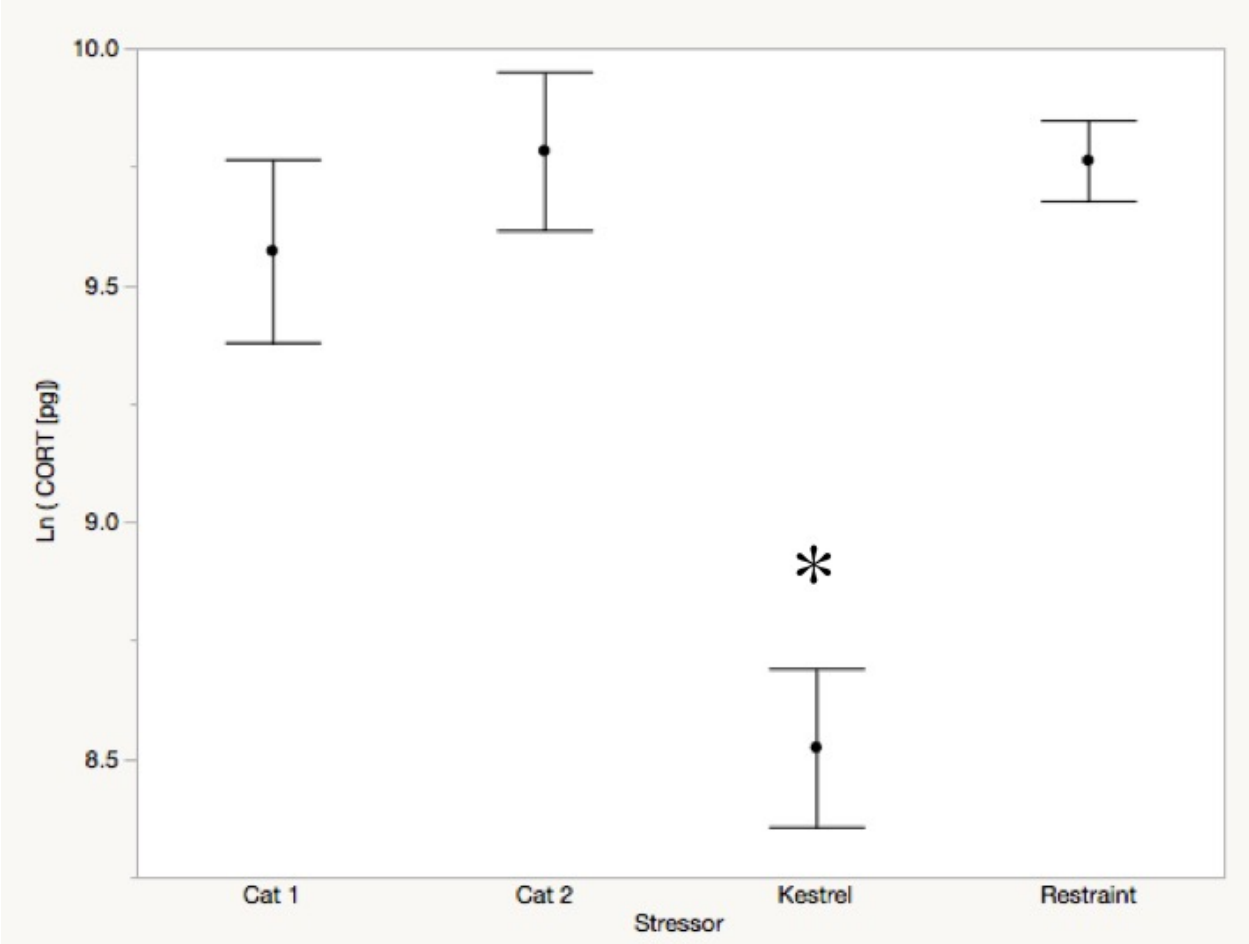

Figure 15. Mean and SEM of CORT response to different stressors in captive male house sparrows. CORT response was significantly lower in response to kestrel exposures as compared to the cat exposures and capture-restraint ($p < .0001$).

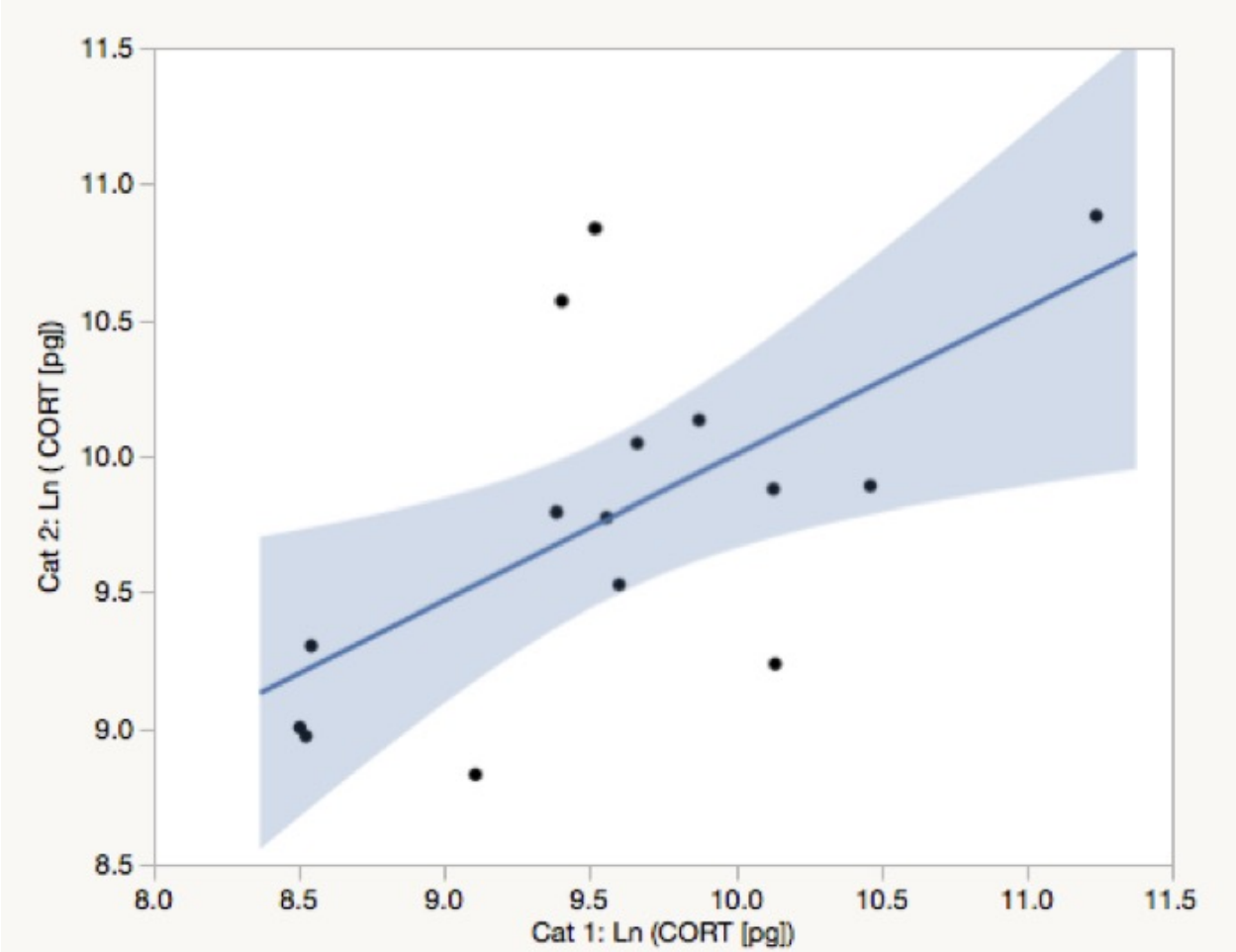

Figure 16. Correlation between CORT response to two cat exposures 35 days apart in captive male house sparrows. Individual CORT response was positively correlated between the two exposures (R^2 = .62, p = .014).

Table 3. Correlations between CORT levels in pre-experiment baseline, first cat exposure (cat 1), later cat exposure (cat 2), kestrel exposure, and capture-restraint in captive male house sparrows.

Sample	Cat 1	Cat 2	Kestrel	Restraint
Pre-experiment baseline	**R^2 = 0.63 p = 0.021**	R^2 = 0.46 p = 0.12	R^2 = 0.10 p = 0.75	R^2 = 0.50 p = 0.083
Cat 1		**R^2 = 0.62 p = 0.014**	**R^2 = 0.66 p = 0.021**	R^2 = 0.42 p = 0.147
Cat 2			R^2 = 0.50 p = 0.097	**R^2 = 0.61 p = 0.021**
Kestrel				R^2 = 0.23 p = 0.473

P-values below 0.05 are indicated in bold.

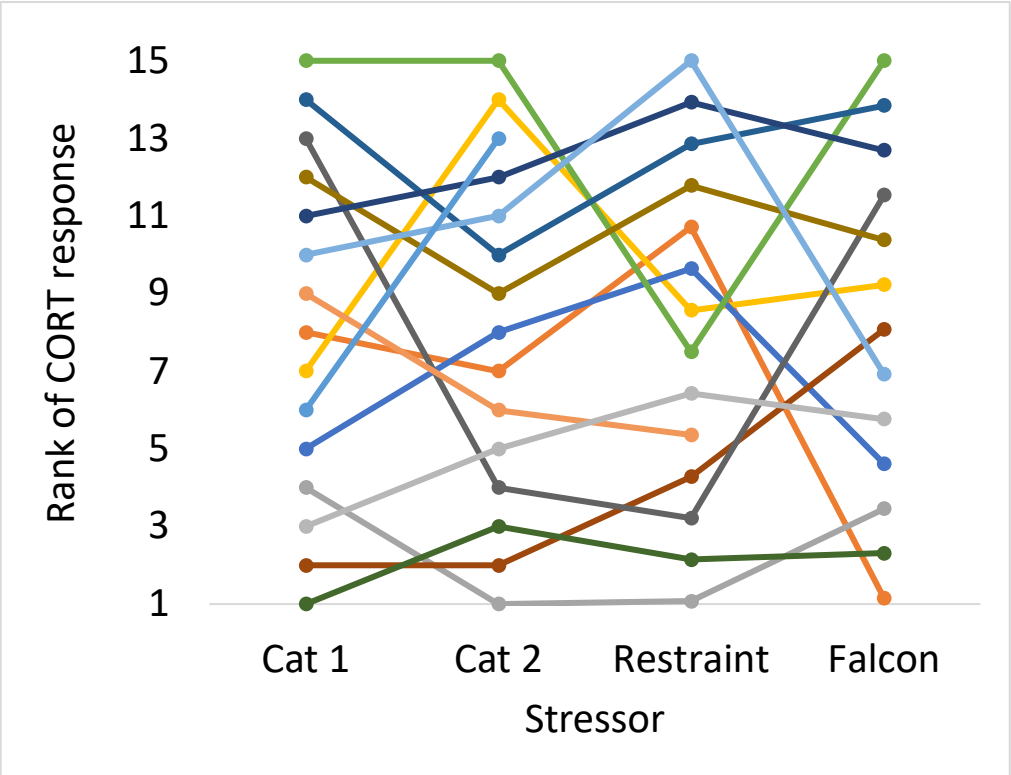

Figure 17. Rank order of CORT response to different predators in captive male house sparrows. Due to the loss of two birds later in the experiment, rank order for capture-restraint and falcon samples are corrected (corrected rank = [rank / n]*15).

Discussion

Much of our understanding of individual variation in stress sensitivity in birds comes from studies utilizing the standardized capture-restraint protocol. It is frequently used as a simple field measure to approximate an individual's responsiveness to stressors in general. As a standardized method, capture-restraint is very useful for comparing between species, between studies, and for repeated measures of individuals across time. However, few studies have considered the relationship between individual response to the capture-restraint protocol and an ecologically-relevant stressor. In fact, how birds respond to different stressors or different types of stressors has been the subject of very little attention.

We measured CORT response to three different stressor types (capture-restraint, live cat, and live kestrel), and found that magnitude of CORT response varies by stressor type. In this

experiment, captive house sparrows responded to cat exposure and to capture-restraint with a similar magnitude of circulating CORT elevation. Interestingly, their response to kestrel exposure was lower, but they still mounted a significant CORT response compared to baseline. Previous studies on captive passerine CORT response to predators have suggested that predator exposure elicits a higher stress response than capture-restraint (Canoine et al. 2013) or non-predator object presentation (Cockrem and Silverin 2002a). Our results suggest a different pattern: CORT was highest in response to capture-handling and cat exposure, and lower in response to kestrel exposure. However, unlike our experiment, both previous studies did not use repeated measures design, had smaller sample sizes, and were conducted in different passerine species (European stonechats and great tits). In our study, both predators were live and acted threateningly toward the caged birds, and are known wild predators of house sparrows where they co-occur. These results suggest that sparrows' magnitude of CORT response depends on the stressor type. In this case, it is possible that house sparrows responded less strongly to the kestrel than to the cat because cats are more abundant than small raptors in their suburban habitat; frequent exposure to a common predator may prime them to respond quickly and strongly to future exposures in order to maximize survival.

Individual response was often correlated between stressors, but this is not entirely consistent across the experiment. For example, CORT response to the first cat exposure was positively correlated with both the second cat exposure sampling event and with the falcon exposure sampling event, but not with the capture-restraint sampling event. However, capture-restraint CORT was positively correlated with the second cat exposure sampling event, but not with the falcon sampling event. In spite of this variation in correlated responses, individual CORT response was repeatable across all four stressor samples. These results suggest that

although magnitude of stress response may differ between stressor types, overall an individual's sensitivity to stress is consistent. Due to the pattern of positive correlations in CORT response between sampling events, it seems likely that our small sample size led to non-significant results in some correlations.

Similar to our findings, one previous study in great tits reported that maximal CORT in response to capture-restraint protocol was repeatable within individuals across multiple exposures (Cockrem and Silverin 2002b). The current results indicate that mean CORT response to live cat exposures 35 days apart do not differ, and individual CORT response is correlated between the two exposures. Together, these studies suggest that individual passerine response to a particular stressor is consistent across exposures, at least in controlled environments.

An individual's behavioral response to stressors is often consistent across time, so understanding how they respond in one situation provides information about how they respond in other contexts. These correlated responses are called behavioral syndromes, and have been well studied in birds (Sih et al. 2004; Cockrem 2007). The behavioral syndromes related to fear responses are often termed "proactive" and "reactive," and are associated with higher fitness in predictable and unpredictable environments respectively (Cockrem 2007). It is less clear whether these behavioral syndromes also encompass CORT response to stressors in birds. It is hypothesized that reactive birds should have higher CORT responses than proactive birds, and that this should be consistent across time and conditions (Cockrem 2007). Here, we show that individual CORT response to one predator is consistent across time, supporting the idea that CORT response underlies proactive vs. reactive behavioral syndromes. We also find that individuals tend to respond similarly across stressor types, though magnitude of response may be somewhat context-dependent.

References

Bonnington C, Gaston KJ, Evans KL. 2013. Fearing the feline: Domestic cats reduce avian fecundity through trait-mediated indirect effects that increase nest predation by other species. J. Appl. Ecol. 50:15–24.

Breuner CW, Orchinik M. 2001. Seasonal regulation of membrane and intracellular corticosteroid receptors in the house sparrow brain. J. Neuroendocrinol. 13:412–420.

Butler LK, Bisson IA, Hayden TJ, Wikelski M, Romero LM. 2009. Adrenocortical responses to offspring-directed threats in two open-nesting birds. Gen. Comp. Endocrinol. 162:313–318.

Canoine V, Hayden TJ, Rowe K, Goymann W. 2013. The Stress Response of European Stonechats Depends on the Type of Stressor. Behaviour 139:1303–1311.

Churcher PB, Lawton JH. 1987. Predation by domestic cats in an English village. J. Zool. 212:439–455.

Cockrem JF. 2007. Stress, corticosterone responses and avian personalities. J. Ornithol. 148:169–178.

Cockrem JF, Silverin B. 2002a. Sight of a predator can stimulate a corticosterone response in the great tit (Parus major). Gen. Comp. Endocrinol. 125:248–255.

Cockrem JF, Silverin B. 2002b. Variation within and between birds in corticosterone responses of great tits (Parus major). Gen. Comp. Endocrinol. 125:197–206.

Gentle LK, Kettel EF. 2013. Attempted predation by Common Kestrel at a House Sparrow nestbox. Br. Birds.

Kübler S, Kupko S, Zeller U. 2005. The kestrel (Falco tinnunculus L.) in Berlin: Investigation of breeding biology and feeding ecology. J. Ornithol. 146:271–278.

Kuhlman JR, Martin LB. 2010. Captivity affects immune redistribution to skin in a wild bird. Funct. Ecol. 24:830–837.

Landys MM, Ramenofsky M, Wingfield JC. 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. Gen. Comp. Endocrinol. 148:132–149.

Lendvai AZ, Giraudeau M, Chastel O. 2007. Reproduction and modulation of the stress response: an experimental test in the house sparrow. Proc. R. Soc. B Biol. Sci. 274:391–397.

Lindstrom K, Hasselquist D, Wikelski M. 2005. House sparrows (Passer domesticus) adjust their social status position to their physiological costs. Horm. Behav. 48:311–320.

Marasco V, Boner W, Heidinger B, Griffiths K, Monaghan P. 2015. Repeated exposure to stressful conditions can have beneficial effects on survival. Exp. Gerontol. 69:170–175.

Martin LB, Kidd L, Liebl AL, Coon CAC. 2011. Captivity induces hyper-inflammation in the house sparrow (Passer domesticus). J. Exp. Biol. 214:2579–2585.

Monaghan P. 2014. Organismal stress, telomeres and life histories. J. Exp. Biol. 217:57–66.

Romero LM, Romero RC. 2002. Corticosterone Responses in Wild Birds : The Importance of Rapid Initial Sampling. Condor 104:129–135.

Sapolsky RM, Romero LM, Munck AU. 2000. How Do Glucocorticoids Influence Stress Responses ? Endocr. Rev. 21:55–89.

Scheuerlein A, Van't Hof T, Gwinner E. 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (Saxicola torquata axillaris). Proc. R. Soc. B Biol. Sci. 268:1575–1582.

Schielzeth H, Nakagawa S. 2011. rptR: Repeatability for Gaussian and non-Gaussian data. R Package.

Sih A, Bell A, Johnson JC. 2004. Behavioral syndromes: An ecological and evolutionary overview. Trends Ecol. Evol. 19:372–378.

Silverin B. 1998. Behavioural and hormonal responses of the pied flycatcher to environmental stressors. Anim. Behav. 55:1411–1420.

Wingfield JC. 1994. Modulation of the adrenocortical response to stress in birds. In: Davey, K.G., Peter, R.E., Tobe SS, editor. Perspectives in Comparative Endocrinology. p. 520–528.

Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M, Richardson RD. 1998. Ecological Bases of Hormone-Behavior Interactions : The "Emergency Life History Stage." 38:191–206.

Wingfield JC, Sapolsky RM. 2003. Reproduction and Resistance to Stress: When and How. J. Neuroendocrinol. 15:711–724.

Wingfield JC, Smith JP, Farner DS. 1982. Endocrine Responses of White-Crowned Sparrows to Environmental Stress. 84:399–409.

Wingfield JC, Vleck CM, Moore MC. 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. Endocrinology 264:419–428.

Woods M, Mcdonald RA, Harris S. 2003. Predation of wildlife by domestic cats Felis catus in Great Britain. Mamm. Rev. 33:174–188.

Yalden DW. 1980. Notes on the diet of urban kestrels. Bird Study 27:235–238.

CHAPTER 7: CONCLUSION AND FUTURE DIRECTIONS

Telomere loss occurs throughout life due to accumulating oxidative damage and normal DNA replication processes (Monaghan 2014; Zhao et al. 2014; Haussmann and Heidinger 2015). Stress exposure has been shown to accelerate telomere loss and reduce longevity, particularly when stress is experienced in early life (Epel et al. 2004; Haussmann and Marchetto 2010; Tyrka et al. 2010; Herborn et al. 2014; Asghar et al. 2015). The impact of stress experience on telomere dynamics during adulthood is less studied, but there is evidence that stress can accelerate telomere loss in adult birds (Monaghan et al. 2012; Hau et al. 2015). Elevation of glucocorticoid hormones in response to stress, including corticosterone, leads to increased oxidative damage (Costantini et al. 2011; Monaghan 2014). Therefore, individual differences in telomere loss rate may be partially mediated by differences in sensitivity to stress.

Telomere length is most commonly measured in blood (leukocytes in mammals, erythrocytes in birds) as it is an easy tissue to sample noninvasively and has high cellular turnover (Haussmann et al. 2007). Few studies have compared telomere length between tissues in birds, and in house sparrows it is unknown whether telomere length is correlated between tissues (Schmidt et al. 2016). Sperm telomere length is of particular interest as direct inheritance of germ line telomeres is one hypothesized pathway of telomere inheritance (Haussmann and Heidinger 2015). Gonadal tissue is highly proliferative and also expresses telomerase throughout life in short-lived birds (Haussmann et al. 2007). Sperm are highly susceptible to oxidative damage, but upregulated telomerase expression in the gonad may protect gamete telomeres against damage caused by increased oxidative stress (Almbro et al. 2011; Losdat et al. 2011; Haussmann and Heidinger 2015).

My research was motivated by one complex question: does paternal stress experience impact offspring aging and longevity via sperm telomere dynamics? This contains aspects of parental effects, life history, telomere biology, and mechanisms of inheritance. To approach this question, I primarily focused on natural variation in and sensitivity to stress of sperm telomere dynamics in house sparrows.

In Chapter 2, I modified a commercial DNA extraction kit protocol for use in isolating DNA from avian sperm and validated this method in house sparrows. I also describe in detail how to perform cloacal massage on passerines, and this methods publication was accompanied by a video description so that learning this technique will be more accessible to others interested in answering questions about sperm.

In Chapter 3, I examined relationships between blood and sperm telomere length, and father and offspring telomere length, in free-living house sparrows. I found that, within individuals, blood and sperm telomere length were positively correlated within individuals and that sperm telomeres were longer than blood telomeres. I also found that sperm telomere length varied across the season, but in a small sample, no relationship between father and offspring telomere length. These results suggest that sperm telomere length may decline with age in birds, as does blood telomere length. Interestingly, previous work in humans has found that older men have longer sperm telomeres, but this is the first time sperm telomere length has been measured in birds (Kimura et al. 2008; Aston et al. 2012; Turner and Hartshorne 2013). These results further suggest that sperm are afforded additional telomere-protective measures as compared to blood, but that sperm telomeres are still sensitive to environmental or internal influences.

Elevated expression of telomerase in gonads likely contribute to the finding of relatively long sperm telomeres. However, the variation in sperm telomere length across the season shows

that telomerase does not inhibit change in telomere length; how sperm telomeres change with season may be related to relationships between telomerase and circulating testosterone or corticosterone, both of which are involved in mediating reproductive cycles (Young and Nelson 2001; Wingfield and Sapolsky 2003). I found no relationship between paternal sperm telomere length and offspring early life telomere length, but, due to small sample size, feel that this is not a complete answer. Previous studies in birds have found relationships between both maternal and paternal blood telomere length and offspring telomere length, which we also did not observe (Horn et al. 2011; Asghar et al. 2014; Reichert et al. 2015). While the inheritance of telomere length is not well understood, it is likely complicated; paternal sperm telomere length may be only partially responsible for offspring early life telomere length if at all, and therefore more and larger studies are needed to tease apart the relative contributions of maternal, paternal, and developmental mechanisms.

In Chapters 4 and 5, I measured telomere responses to stress exposure in captive sparrows. Stressor type and duration varied between these two experiments, but in both cases sperm and blood telomere length did not differ between the stress and control treatments. However, I found that while sperm and blood telomere length were still positively correlated within individuals, sperm telomere length was not longer than blood telomere length, contrary to my findings in free-living birds (Figure 18). It is important to note that telomere length cannot be directly compared between these studies due to use of different reference samples, but in Figure 18 I arbitrarily set blood telomere length at 1 unit to illustrate the differences in relative sperm telomere length by captivity status and duration. Evidence from my captive studies and others suggest that captivity alone induces chronic stress in house sparrows, so this finding indicates that chronic stress may impact sperm telomere length, but this impact may only occur over a

longer time scale than I originally predicted (Rich and Romero 2001; Kuhlman and Martin 2010; Martin et al. 2011). In Chapter 4 I also found that sperm telomere length is less repeatable over a medium time scale than is blood telomere length over a relatively long period. Together, these findings further support the hypothesis that sperm telomere length is changing over time and may be sensitive to environmental factors such as stress exposure. While this is very likely mediated by oxidative damage, to which sperm are particularly susceptible, it is not clear if CORT is involved in mediating the link between stress exposure and oxidative damage (Almbro et al. 2011; Losdat et al. 2011).

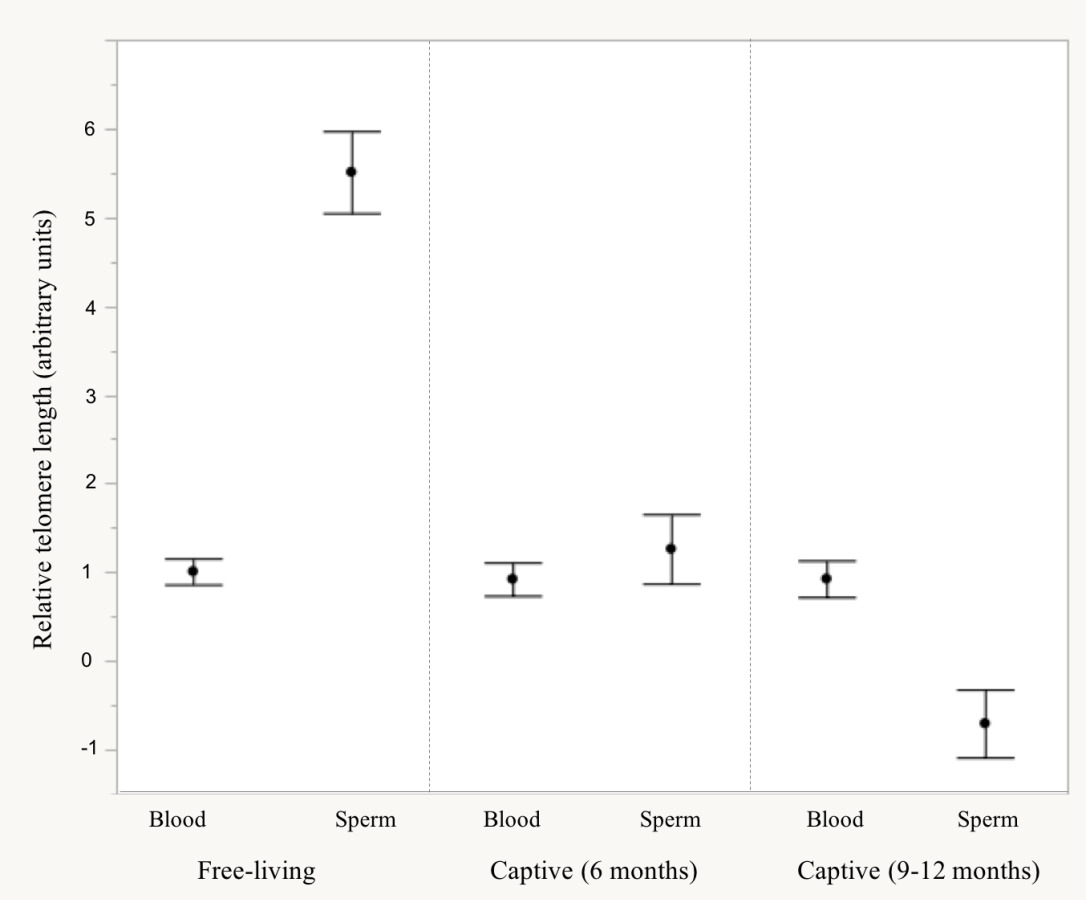

Figure 18. Mean and SEM of relative telomere length in blood and sperm of free-living and captive male house sparrows. Dotted lines indicate where telomere length cannot be directly compared due to use of different reference samples. Mean blood telomere length for each group was set at 1 arbitrary unit to illustrate the differences in relationship between blood and sperm telomere length across captivity status and duration.

In Chapter 6, I measured CORT responses to multiple stressors in captive house sparrows. I was interested in the context-dependent nature of stress responses, and how an individual's response to one stressor informs us about its response to other, ecologically-relevant stressors. I found that individual response to one predator is consistent across exposures and is likely indicative of response to another predator. These birds' response to the predators may be influenced by their experiences; in areas where I have seen house sparrows living around Fargo, cats are more common than falcons, so birds living in this area may be primed through exposure to respond strongly to cat presence. This study highlights the need for studies involving multiple types of stress (as in Chapter 4 versus Chapter 5).

This dissertation provides baseline knowledge of sperm telomere biology in passerines; future studies will be needed to improve our understanding of the relationship between stress and sperm telomere dynamics in various contexts and address the role of sperm telomere dynamics in shaping offspring aging. The opportunities for such studies are vast, and I have several ideas for future approaches to these questions.

We do not fully understand how stress experienced during adulthood impacts telomere dynamics in birds, but stress experienced during development has repeatedly been linked to accelerated telomere loss and reduced longevity (Haussmann and Marchetto 2010; Monaghan et al. 2012; Herborn et al. 2014; Monaghan 2014). The question of how stress impacts sperm telomere dynamics could be addressed by rearing chicks in captivity under stress and control treatments and maintaining them in an aviary for one or more years. This design would allow for comparisons of blood and sperm telomere length across time and in relation to developmental stress, as well as providing an opportunity for following individual patterns in circulating hormones, ejaculate volume, sperm quality, and sperm telomere length across a breeding season.

Captive Japanese quail and great tits have been artificially selected for high-stress and low-stress lines, and these populations would provide an invaluable opportunity to compare CORT response, oxidative stress, testicular telomerase, and sperm telomere dynamics across time. Exposing these birds to repeated stressors and comparing between lines would highlight the role of CORT in mediating telomere loss. Wild-caught house sparrows may not be the best model for a captive breeding project of this scale, but a similar experiment could be conducted in any number of birds that are amenable to captivity.

While I did not find differences in stress exposure between captive house sparrows in control and stress treatments, this type of experiment could be conducted in free-living sparrows in our population. In fact, I attempted to induce stress in free-living males by injecting them with keyhole limpet hemocyanin (KLH), a protein derived from shellfish which activates adaptive immune responses in birds. KLH has been used to induce antigen responses in house sparrows for 10 days or longer (Lee et al. 2006; Martin et al. 2006; Pap et al. 2010). Unfortunately, I was unable to recover any of the 40 birds injected with KLH or vehicle in 2016. These birds were caught by mist netting near nest boxes very early in the breeding season before males were committed to nest sites. Recovery of experimental birds could be improved by capturing them on the nest box shortly after the first clutch is laid and removing the eggs. A similar experimental design could be implemented with CORT implants or a vector-borne infection such as malaria, which accelerated telomere loss in reed warblers (Asghar et al. 2015). Most birds should re-nest and would thus be available for repeat sampling of blood and sperm telomeres. This would also be an opportunity to compare early life telomere dynamics in chicks with stressed and control fathers.

This research shows that sperm telomere length is likely susceptible to change across relatively long time frames (months or longer), and suggests that chronic stress may be involved in sperm telomere attrition. While my findings on the relationship between paternal sperm telomere length and offspring early life telomere dynamics were inconclusive, the role of gamete telomere dynamics in shaping offspring aging warrants further investigation. Further, sperm telomeres may be protected from damage in response to acute stress, and more work is needed to determine the mechanisms of telomere damage and protection in both somatic and germline tissues. The effects of parental stress exposure on offspring fitness are not well understood, particularly from a mechanistic standpoint; understanding how stress impacts gamete telomere dynamics and the contribution of gamete telomeres on offspring early life telomere dynamics will inform a wide range of fields in biology and biomedicine.

References

Almbro M, Dowling DK, Simmons LW. 2011. Effects of vitamin E and beta-carotene on sperm competitiveness. Ecol. Lett. 14:891–895.

Asghar M, Bensch S, Tarka M, Hansson B, Hasselquist D. 2014. Maternal and genetic factors determine early life telomere length. Proc. R. Soc. B Biol. Sci. 282:20142263.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science 347:436–438.

Aston KI, Hunt SC, Susser E, Kimura M, Factor-litvak P, Carrell D, Aviv A. 2012. Divergence of sperm and leukocyte age-dependent telomere dynamics: Implications for male-driven evolution of telomere length in humans. Mol. Hum. Reprod. 18:517–522.

Costantini D, Marasco V, Møller AP. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181:447–456.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. 101:17312–17315.

Hau M, Haussmann MF, Greives TJ, Matlack C, Costantini D, Quetting M, Adelman JS, Miranda AC, Partecke J. 2015. Repeated stressors in adulthood increase the rate of biological ageing. Front. Zool. 12:1–10.

Haussmann MF, Heidinger BJ. 2015. Telomere dynamics may link stress exposure and ageing across generations. Biol. Lett. 11:20150396.

Haussmann MF, Marchetto NM. 2010. Telomeres: Linking stress and survival, ecology and evolution. Curr. Zool. 56:714–727.

Haussmann MF, Winkler DW, Huntington CE, Nisbet ICT, Vleck CM. 2007. Telomerase activity is maintained throughout the lifespan of long-lived birds. Exp. Gerontol. 42:610–618.

Herborn KA, Heidinger BJ, Boner W, Noguera JC, Adam A, Daunt F, Monaghan P. 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. Proc. R. Soc. B Biol. Sci. 281:20133151.

Horn T, Robertson BC, Will M, Eason DK, Elliott GP, Gemmell NJ. 2011. Inheritance of telomere length in a bird. PLoS One 6:1–5.

Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, Cupples A, Hunkin JL, Gardner JP, Lu X, et al. 2008. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 4:1–9.

Kuhlman JR, Martin LB. 2010. Captivity affects immune redistribution to skin in a wild bird. Funct. Ecol. 24:830–837.

Lee KA, Martin LB, Hasselquist D, Ricklefs RE, Wikelski M. 2006. Contrasting adaptive immune defenses and blood parasite prevalence in closely related Passer sparrows. Oecologia 150:383–392.

Losdat S, Richner H, Blount JD, Helfenstein F. 2011. Immune activation reduces sperm quality in the great tit. PLoS One 6.

Martin LB, Hasselquist D, Wikelski M. 2006. Investment in immune defense is linked to pace of life in house sparrows. Oecologia 147:565–575.

Martin LB, Kidd L, Liebl AL, Coon CAC. 2011. Captivity induces hyper-inflammation in the house sparrow (Passer domesticus). J. Exp. Biol. 214:2579–2585.

Monaghan P. 2014. Organismal stress, telomeres and life histories. J. Exp. Biol. 217:57–66.

Monaghan P, Heidinger BJ, D'Alba L, Evans NP, Spencer KA. 2012. For better or worse: reduced adult lifespan following early-life stress is transmitted to breeding partners. Proc. R. Soc. B Biol. Sci. 279:709–714.

Pap PL, Czirják GÁ, Vágási CI, Barta Z, Hasselquist D. 2010. Sexual dimorphism in immune function changes during the annual cycle in house sparrows. Naturwissenschaften 97:891–901.

Reichert S, Rojas ER, Zahn S, Robin J-P, Criscuolo F, Massemin S. 2015. Maternal telomere length inheritance in the king penguin. Heredity (Edinb). 114:10–16.

Rich E, Romero L. 2001. Daily and photoperiod variations of basal and stress-induced corticosterone concentrations in house sparrows (Passer domesticus). J. Comp. Physiol. - B Biochem. Syst. Environ. Physiol. 171:543–547.

Schmidt JE, Sirman AE, Kittilson JD, Clark ME, Reed WL, Heidinger BJ. 2016. Telomere correlations during early life in a long-lived seabird. Exp. Gerontol. 85:28–32.

Turner S, Hartshorne GM. 2013. Telomere lengths in human pronuclei, oocytes and spermatozoa. Mol. Hum. Reprod. 19:510–518.

Tyrka AR, Price LH, Kao H, Marsella SA, Carpenter LL. 2010. Childhood maltreatment and telomere shortening: Preliminary support for an effect of early stress on cellular aging. Biol. Psychiatry 67:531–534.

Wingfield JC, Sapolsky RM. 2003. Reproduction and Resistance to Stress: When and How. J. Neuroendocrinol. 15:711–724.

Young KA, Nelson RJ. 2001. Mediation of seasonal testicular regression by apoptosis. Reproduction 122:677–685.

Zhao Z, Pan X, Liu L, Liu N. 2014. Telomere length maintenance, shortening, and lengthening. J. Cell. Physiol. 229:1323–1329.