Transition Between Phases of the Annual Cycle: Spring Migration to Breeding in Nearctic-Neotropical Songbirds

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ABSTRACT

TRANSITION BETWEEN PHASES OF THE ANNUAL CYCLE: SPRING MIGRATION TO BREEDING IN NEARCTIC-NEOTROPICAL SONGBIRDS

by Kristen Marie Covino

May 2016

Appropriate timing of each life-history stage is crucial for seasonally migratory species. The temporal constraints faced by migratory songbirds require that they overlap preparation for breeding with spring migration. However, previous work has focused primarily on male birds and has produced inconsistent results regarding the degree of overlap between these two life-history stages. I study the degree to which migrating male and female songbirds prepare for breeding throughout spring migration as they move towards their breeding grounds. Overall, male migrants show a significant degree of breeding preparation during spring migration as determined by circulating testosterone levels and their ability to elevate testosterone. Female migrants, on the other hand, did not vary in their degree of breeding preparation throughout the migratory period. That said, in both male and female migrants, some degree of breeding preparation had occurred previous to their passage through my migratory study areas. It is possible that while male migrants continue to prepare for breeding throughout spring migration, female migrants delay the final stages of breeding preparation until they arrive on the breeding grounds.

Since testosterone increases in some birds during the migratory periods and mediates a wide range of effects during the breeding season, I also
investigated whether testosterone was related to migratory stopover biology. To do so I looked for correlates between testosterone and the following measures: likelihood of stopover, stopover duration, level of competition for resources, foraging movement rate, and prey attack rate. However, I was unable to detect any effects of testosterone on the measures of migratory stopover biology that I used in this study. It is possible that since levels are relatively low during migration, testosterone does not directly influence the expression of migratory traits.
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and Appledore Island and members of the Migratory Bird Research Group. The adventures I had with my fellow field researchers at Johnson’s Bayou and Appledore Island will continue to have special meaning to me mostly due to the unique friends I have made. Additionally, Matthew Capps at the Dauphin Island Parks and Beach Board must be acknowledged for his assistance during my fall 2014 field season.

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Finally, I would like to thank my family and friends for their care and support throughout these years. I could not have accomplished my dissertation without the assistance my wife.
DEDICATION

I dedicate my dissertation to my friends, family, and my wife. I owe my sisters, parents, and grandparents a great deal of gratitude for their continuous support. Dr. Sara R. Morris is a consistent source of inspiration and friendship. My conversations and coffee breaks with Dr. Frank R. Moore have enabled me to maintain my sanity throughout these years. Most of all, the support, encouragement, and understanding of my wife and life partner, Amber Bratcher-Covino, have been unwavering throughout my years of graduate work. She is the source of my motivation to do better and to be my very best.

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# TABLE OF CONTENTS

ABSTRACT ......................................................................................................................... ii

ACKNOWLEDGMENTS ......................................................................................................... iv

DEDICATION ....................................................................................................................... vi

LIST OF TABLES ................................................................................................................ xii

LIST OF ILLUSTRATIONS ................................................................................................. xiii

CHAPTER I – GENERAL INTRODUCTION AND SYNOPSIS ........................................ 1

  Testosterone Levels ....................................................................................................... 1
  Hypothalamic-pituitary-gonadal Axis Activity and the Capacity to Elevate Testosterone ....................................................................................................................... 4
  Stopover Biology ............................................................................................................. 6
  Summary .......................................................................................................................... 8
  Conclusions ..................................................................................................................... 9

CHAPTER II – PATTERNS OF TESTOSTERONE IN THREE NEARCTIC-NEOTROPICAL MIGRATORY SONGBIRDS DURING SPRING PASSAGE ..... 13

  Abstract .......................................................................................................................... 13
  Introduction ...................................................................................................................... 13
  Materials and Methods ................................................................................................... 17
  Study Species .................................................................................................................. 17
  Capture and Sampling ..................................................................................................... 18
CHAPTER IV – SEX-SPECIFIC HYPOTHALAMIC-PITUITARY-GONADAL AXIS ACTIVITY IN MIGRATING SONGBIRDS

Abstract........................................................................................................................................... 58

Introduction ......................................................................................................................................... 59

Materials and Methods..................................................................................................................... 63

Study Species and Study Sites .......................................................................................................... 63

Capture and Sampling ....................................................................................................................... 64

Testosterone Assays .......................................................................................................................... 65

Statistical Analyses ............................................................................................................................ 66

Results ............................................................................................................................................... 68

Controls and Time Series Trials ......................................................................................................... 68

Seasonal Variation in GnRH Response ............................................................................................... 69

Temporal Variation .............................................................................................................................. 71

Discussion .......................................................................................................................................... 74
APPENDIX A - Genetic Sexing Protocol For Passerines................................. 106
APPENDIX B - Institutional Animal Care and Use Committee Approval......... 113
APPENDIX C - Federal Banding Permit 1 ...................................................... 114
APPENDIX D - Federal Banding Permit 2 ...................................................... 120
REFERENCES .................................................................................................. 128
LIST OF TABLES

Table 1 Number of bird sampled in Louisiana and Maine during spring migration by age and sex ................................................................. 20

Table 2 Linear mixed model analysis of the effects of age, sex, and sampling location on testosterone ......................................................... 26

Table 3 Linear mixed model analysis of the effects of energetic condition, handling time, sampling date, and time of day on testosterone ............... 28

Table 4 Linear mixed model analysis of testosterone and foraging competition. Testosterone was compared to the total number of other migrants and to the number of migrants within each species’ foraging group (see methods) .......... 29

Table 5 Linear mixed model analyses of circulating testosterone, response to the GnRH injection, and feather hydrogen levels in Black-and-white Warblers ....... 50

Table 6 Linear mixed model analyses of five measures of stopover biology and behavior in Black-and-white warblers during spring migration ...................... 96

Table A1. Stepdown thermocycler settings. ...................................................... 109
LIST OF ILLUSTRATIONS

Figure 1. Theoretical schedule for breeding development in male and female Nearctic-Neotropical migrants. .............................................................. 12

Figure 2. Breeding ranges for three species of Nearctic-Neotropical migrants... 18

Figure 3. Circulating testosterone levels in three species sampled at a southern and a northern site during vernal migration. .................................................... 27

Figure 4. Circulating testosterone levels and corticosterone levels for two species. ............................................................................................................. 29

Figure 5. The breeding range of the Black-and-white Warbler (Mniotilta varia). 43

Figure 6. Time trials for GnRH injections in Black-and-white Warblers. ........... 48

Figure 7. Patterns of testosterone relative to feather hydrogen levels in migrating Black-and-white Warblers................................................................. 49

Figure 8. Testosterone response to gonadotropin-releasing hormone bioassays in migrating Black-and-white Warblers in relation to feather hydrogen isotope ratios........................................................................................................... 52

Figure 9. Pattern of feather hydrogen values relative to passage date in Black-and-white Warblers during spring migration. .............................................. 53

Figure 10. The breeding and wintering range of the Swainson’s Thrush (Catharus ustulatus). .................................................................................................. 67

Figure 11. Time trials for GnRH injections in Swainson’s Thrushes. ................. 69

Figure 12. Testosterone levels in male and female migrating Swainson’s Thrushes before and 30 minutes after an injection of phosphate-buffered saline. ................................................................................................................. 71
Figure 13. Testosterone levels in male migrating Swainson’s Thrushes before and 30 minutes after an injection of GnRH. ................................................................. 72

Figure 14. Testosterone levels in female migrating Swainson’s Thrushes before and 30 minutes after an injection of GnRH. ................................................................. 73

Figure 15. Testosterone response to gonadotropin-releasing hormone bioassays relative to date in migrating Swainson’s Thrushes......................................................... 74

Figure 16. Breeding and wintering range for the Black-and-white Warbler (Mniotilta varia). .................................................................................................................. 86

Figure 17. Testosterone levels in male and female Black-and-white Warblers during spring migration. .............................................................................................................. 94

Figure 18. Testosterone levels at initial capture and upon recapture of Black-and-white Warblers during spring migration. ................................................................. 98

Figure 19. Minimum stopover length in male and female Black-and-white Warblers during spring migration. .............................................................................................. 99

Figure 20. Minimum stopover length in relation to energetic condition in Black-and-white Warblers during spring migration. ......................................................... 100

Figure A1. Photograph of an agarose gel showing two bands for female songbirds and on band for male songbirds................................................................. 112
CHAPTER I – GENERAL INTRODUCTION AND SYNOPSIS

My dissertation research investigates the overlap in aspects of physiology between vernal migration and breeding in Nearctic-Neotropical migrants. The annual cycle of seasonally reproducing animals is divided into a sequence of life-history stages during which different phenotypes are expressed (Jacobs and Wingfield 2000; Piersma and van Gils 2011). According to Finite State Machine Theory there is a limit to the number of traits that can be expressed within an individual at any given stage of the annual cycle (Jacobs and Wingfield 2000; Ramenofsky 2011). Migratory birds provide a rich opportunity to examine the temporal constraints that result in overlap between life-history stages (Ramenofsky and Wingfield 2006). Full life-cycle biology depends essentially on knowing how migrants transition between life-history stages (Marra et al. 2015), yet overlap between stages of the annual cycle are poorly understood. To gain a better understanding of how Nearctic-Neotropical migrants time and regulate these processes, I studied three related aspects of migrating songbirds during spring migration as they approach the breeding grounds: (1) changing levels of testosterone, a hormone that plays an important role in avian breeding biology (Chapters II & III); (2) hypothalamic-pituitary-gonadal axis activity and the ability to elevate testosterone (Chapters III & IV), and (3) the extent to which breeding preparation interacts with migratory stopover biology (Chapter V).

Testosterone Levels

Testosterone plays an important role in breeding biology (Balthazart 1983; Wingfield et al. 1990), influences preparation for migration in both sexes
(Schwabl and Farner 1989a; Deviche 1995; Ramenofsky and Wingfield 2006; Tonra et al. 2011a; Tonra et al. 2011b), and may affect behavior of migrating birds during passage. In my first study (Chapter II), I sampled long-distance, inter-continental migrants at locations that differ dramatically in proximity to the breeding grounds by sampling them in Louisiana where they are relatively far from their destination and in Maine when they are relatively close. I predicted that testosterone levels would be higher in birds sampled closer to their breeding grounds compared to those that were relatively far from their breeding grounds due to the impending onset of breeding. Across all three focal species, males had higher testosterone levels closer to their breeding grounds; however while this relationship was significant in two species (Northern Waterthrush, *Parkesia noveboracensis* and Magnolia Warbler, *Setophaga magnolia*) it was not significant in the Swainson’s Thrush (*Catharus ustulatus*; Covino et al. 2015; Chapter II). Testosterone levels in females did not differ with proximity to the breeding grounds; female Northern Waterthrushes and Swainson’s Thrushes had low levels of testosterone at both locations. Interestingly, however, testosterone levels in female Magnolia Warblers, were relatively high at both locations and similar to levels seen in males at my northern, ‘close’, sampling site.

In an effort to strengthen inferences drawn from the first study (Chapter II), I employed stable hydrogen isotopes from feathers to link individual Black-and-white Warblers (*Mniotilta varia*) captured during vernal migration at a single location to geographically disparate breeding area destinations (Chapter III). I then used these links to investigate the impact of individual variation in proximity
to breeding location on circulating testosterone. This novel approach enabled me
to study migrating individuals from across the entire breeding range of a species
while they passed through a single study site. Males closer to their breeding
grounds had higher testosterone levels than individuals quite distant from their
destination. Again, testosterone levels in females were not related to their
breeding ground proximity.

Some studies have suggested that during the breeding season
corticosterone and testosterone may influence one another (Ketterson et al.
1991; Deviche et al. 2001; Swett and Breuner 2008). Thus, I evaluated the
potential effect of corticosterone on circulating testosterone in both Chapter II
and Chapter III. In all species tested there was no difference in testosterone
levels between birds that had elevated corticosterone levels and those that had
baseline corticosterone levels (Chapter II; Chapter III). These results and those
of other studies (e.g. Wikelski et al. 1999; Devries et al. 2011) suggest that
relatively short handling times (< 30 min) do not significantly influence circulating
testosterone levels.

In the work presented in both Chapter II and Chapter III, testosterone
levels were higher in males when they were closer to their breeding grounds.
Additionally, in both studies variation in testosterone was not explained by
relative distance to the breeding grounds in female migrants. There was no
difference in the testosterone levels between older and younger birds in any of
the three species tested in Chapter II. However, in male- but not female- Black-
and-white Warblers, older birds had higher testosterone than younger birds
(Chapter III). It is possible that, because young birds are undertaking both spring migration and preparation for breeding for the first time, their schedule for doing so lags behind that of older birds; however, this pattern was only seen in one of four species studied. My studies indicate that male migrants overlap the developmental phase of breeding with spring migration by increasing their testosterone levels as they approach the breeding grounds. This overlap presumably enables males to transition smoothly from migration behaviors to breeding behaviors (e.g. territory establishment) soon after arrival on the breeding grounds. Conversely, female migrants do not appear to overlap the developmental phase of breeding with migration, at least as determined by their lack of increases in testosterone levels.

Hypothalamic-pituitary-gonadal Axis Activity and the Capacity to Elevate Testosterone

While many studies have investigated seasonal levels of circulating testosterone as a measure of breeding preparation, we must also consider the potential of individuals to elevate testosterone, termed $R_{potential}$ (Goymann et al. 2007). It is likely that full activation of the hypothalamic-pituitary-gonadal (HPG) axis and resulting gonadal recrudescence take several weeks. These processes must take place either before or during spring migration in order for migratory birds to ensure that they are prepared for breeding activities soon after arrival on the breeding grounds. Additionally, while testosterone levels may also increase during this time, some of the effects of high testosterone levels may be antagonistic to migration. Thus I predicted that, since both males and females
must be able to quickly transition to breeding activities once they reach their
destination, both sexes increase their ability to elevate testosterone (R\text{potential}) as
they approach the breeding grounds as a part of this transition. To test this
prediction I employed a field bioassay where an injection of gonadotropin-
releasing hormone stimulates the reproductive pathway and tests the R\text{potential} of
the gonads. For this aspect of my dissertation, I used both a comparative
approach by studying the Swainson’s Thrush at migration sites relatively near
and far from their breeding grounds (Chapter IV) and a study design which
allowed me to investigate linear patterns across breeding ground proximity by
studying the Black-and-white Warbler (Chapter III).

Male Swainson’s Thrushes sampled closer to their breeding grounds
showed a much greater R\text{potential} compared to males further from their breeding
grounds. Although throughout the migratory period, R\text{potential} among females was
higher than circulating levels of testosterone, R\text{potential} was similar across sites. In
Black-and-white Warblers, R\text{potential} was higher in males that were closer to their
breeding grounds but there was no pattern between R\text{potential} and breeding ground
proximity in females. These patterns mirrored the patterns of circulating
testosterone relative to breeding ground proximity in male and female Black-and-
white Warblers.

Taken together, these studies indicate that the ability to elevate
testosterone, R\text{potential}, increases in male migrants as they move closer to their
breeding destination. Additionally, the increases seen in R\text{potential} outpace
increases in circulating testosterone levels. Males are overlapping the
development of breeding throughout spring migration as measured by both circulating testosterone levels (Chapters II & III) and their ability to elevate testosterone ($R_{\text{potential}}$, Chapters III & IV). In females, $R_{\text{potential}}$ was unrelated to breeding ground proximity in both studies. That said, most females tested were able to elevate testosterone above circulating levels. Additionally, although the sample size was small, females sampled during fall migration were not able to elevate testosterone above circulating levels (Chapter IV). This seasonal difference may indicate that in females some amount of hypothalamic-pituitary-gonadal axis activation does occur prior to arrival on the breeding grounds and it may occur early in spring migration or on the wintering grounds prior to departure. It is possible that females begin the development of breeding early and delay the final stages of follicular development until they receive supplementary (social or environmental) cues upon arrival on the breeding grounds.

Stopover Biology

In my last chapter, I explore how preparation for breeding might influence the biology of birds during migration (Chapter V). The majority of time and energy spent throughout migration is spent on stopover rather than migratory flight (Hedenstrom and Alerstam 1997), and performance on stopover may determine the success of the overall journey. Key determinants of the reproductive success of migratory birds are arrival timing on the breeding grounds and arrival condition (Moore et al. 2005). Testosterone is known to mediate several behaviors during the breeding season (Balthazart 1983), and it may influence other aspects of
stopover biology during spring migration as well. I investigated the relationship between testosterone and the following aspects of stopover biology: likelihood of stopover, stopover duration, level of competition for resources, foraging movement rate, and prey attack rate. Since testosterone levels may vary daily as well as seasonally, I also investigate whether testosterone levels change during the stopover period in a subset of recaptured birds.

Testosterone levels were lower in birds on the day of recapture compared to what they were at initial capture 2-14 days prior (Chapter V). This result is interesting and ran contrary to my prediction that testosterone levels may be low at first capture as a result of suppression of the hypothalamic-pituitary-gonadal axis during the migratory flight.

Testosterone was unrelated to all the measures of stopover biology that I investigated (Chapter V). While the range of testosterone levels in this study was broad, levels in both males and females were on the low end for what has been reported in breeding songbirds (reviewed in Ketterson et al. 2005). It is possible that because testosterone is found at relatively low levels during spring migration, testosterone does not play a primary role in mediating behaviors during this phase of the annual cycle. Additionally, given the inherent variation associated with evaluating naturally occurring hormone levels, this study design may have be inadequate at detecting what could be relatively weak relationships between T and the measures of stopover biology and behavior that I evaluated.

Contrary to my findings, several studies suggest that testosterone may play a primary role in organizing both behavioral and physiological aspects of
developing and expressing migratory traits (Stetson and Erickson 1972; Schwabl and Farner 1989a; Schwabl and Farner 1989b; Ramenofsky and Wingfield 2007; Tonra et al. 2011a). However, some current research suggests that testosterone may only play a ‘permissive’ role in organizing the development of the migratory phenotype (Wang et al. 2013) and that the timing of hormonal changes (e.g. increases in testosterone) must be considered as well (Ramenofsky and Németh 2014). Although logistically difficult, future studies investigating the role of testosterone in migrating birds should employ the use of manipulation experiments (e.g. implants) in free-living individuals. This study design would remove the inherent variation in testosterone levels and will allow researchers the comparative power to investigate these relationships in a natural setting. Additionally, this approach would allow the flexibility to investigate both the levels of hormones and timing of hormonal changes that may be necessary to organize expression of migratory traits.

**Summary**

My dissertation research demonstrates the following:

1. Male migrants increase their production of circulating testosterone as well as their ability to elevate testosterone, $R_{potential}$, during spring migration (Chapters II-IV). This indicates a substantial degree of overlap between spring migration and breeding development in male Nearctic-Neotropical migrants.

2. Neither circulating testosterone levels nor $R_{potential}$ change during spring migration in female songbirds (Chapters II-IV). That said, there is evidence
that some degree of breeding development occurs either before or during early spring migration: females can elevate testosterone above circulating levels during spring migration, and their capacity to elevate testosterone is higher in spring compared to fall.

3. Circulating testosterone is not directly related to the measures of stopover biology that I investigated (Chapter V). While indirect, there is weak evidence for a link between testosterone, stopover length, and foraging rates (see Chapter V).

4. The peak response to an injection of GnRH, as determined by elevations in testosterone levels, occurs at 30 minutes post injection in migrating songbirds (Chapters III & IV). This is similar to previous studies of breeding and non-breeding (wintering) songbirds.

5. Circulating testosterone is not affected by high corticosterone levels when passively captured during migration (Chapters II & III). However, researchers should exhibit caution since there is some indication that handling time can negatively affect circulating testosterone levels (Chapter II).

Conclusions

The findings of the first two components of my research allow me to conclude that for male long-distance, inter-continental migrants the developmental phase of breeding begins prior to and continues during spring migration (Chapters II-IV). My findings are similar to previous studies of both short-distance (*Zonotrichia leucophrys pugetensis*; Wingfield and Farner 1978a)
and medium to long-distance intra-continental migrants (Z. l. gambelii; Wingfield and Farner 1978b). No study previous to Covino et al. (2015) has demonstrated that testosterone levels increase during spring migration in a long-distance inter-continental songbird. Bauchinger et al. (2007) failed to detect any increase in testosterone in Garden Warblers (Sylvia borin), a long-distance inter-continental migrant, but demonstrated increases in testis size during migration (Bauchinger et al. 2005; Bauchinger et al. 2007). Additionally, Tonra et al. (2013) detected increasing testosterone levels late in the wintering period in male American Redstarts (Setophaga ruticilla), also a long-distance inter-continental migrant. These results are similar to my GnRH results (Chapters III & IV), and also indicate that at least some breeding preparation occurs prior to the beginning of spring migration. By combining my research with previous studies, I suggest the relationship depicted in Figure 1 as a general schedule for breeding development in male long-distance, inter-continental migrants.

In my studies, female long-distance, inter-continental migrants did not appear to be preparing for breeding during spring migration (Chapters II-IV). My findings are similar to the Wingfield and Farner (1978a) study of the short-distance subspecies of an intra-continental migrant, the White-crowned Sparrow (Z. l. pugetensis) where testosterone levels also did not change in females during spring migration. However, Wingfield and Farner (1978b) did detect increases in testosterone in a separate study of the medium/long-distance White-crowned Sparrow conspecific (Z. l. gambelii). Additionally, in these same studies estradiol levels were found to increase in both subspecies during this time (Wingfield and
Farner 1978a; Wingfield and Farner 1978b). Further, these studies have detected measurable ovarian development during spring migration but development appears to be limited and yolking of the follicles is delayed until after arrival on the breeding grounds (Wingfield and Farner 1978a; Wingfield and Farner 1978b). My findings indicate that females can elevate testosterone during spring migration but that their $R_{potential}$ does not change during the migratory period (Chapters III & IV). Based on my work and that of previous studies, I propose that female long-distance, inter-continental migrants begin breeding development prior to or early during spring migration but that development does not continue throughout the migratory period and the final stages for of breeding development are delayed until arrival on the breeding grounds (Figure 1).
Figure 1. Theoretical schedule for breeding development in male and female Nearctic-Neotropical migrants.

Note: Males begin breeding development prior to departure on spring migration and continue this process throughout the migratory period. Females breeding development in late winter and/or early during spring migration but delay the final stages until arrival on the breeding grounds.
CHAPTER II – PATTERNS OF TESTOSTERONE IN THREE NEARCTIC–NEOTROPICAL MIGRATORY SONGBIRDS DURING SPRING PASSAGE


Abstract

Preparation for breeding may overlap extensively with vernal migration in long-distance migratory songbirds. Testosterone plays a central role in mediating this transition into breeding condition by facilitating changes to physiology and behavior. While changes in testosterone levels are well studied in captive migrants, these changes are less well known in free-living birds. I examined testosterone levels in free-living Nearctic-Neotropical migrants of three species during their vernal migration. Testosterone levels increased during the migratory period in males of all three species but significantly so in only two. Testosterone levels in females remained the same throughout their migration. My results support the extensive overlap between vernal migration and breeding preparation in male songbirds. The pattern of testosterone changes during vernal migration is far from clear in females.

Introduction

Animals express different phenotypes at different times of the year as they transition through their annual cycle. Piersma and van Gils (2011) purport that life-history stages are the specific phenotypic periods that exist within a single individual at different points during the annual cycle. Finite State Machine Theory
posits that there is a limit to the number of behavioral, physiological, and morphological traits that can be expressed within an individual at any time (Jacobs and Wingfield 2000). Accordingly physiological tradeoffs must occur as an organism transitions between states, i.e. stages in the annual cycle (Jacobs and Wingfield 2000; Ramenofsky 2011). Migratory songbirds typically exhibit the following series of annual life-history stages: breeding, pre-basic molt, autumn migration, overwintering, and vernal migration; and some species also complete a pre-alternate molt prior to vernal migration (sensu Jacobs and Wingfield, 2000). The expression of traits associated with these different life-history stages of migratory species must coincide due to temporal constraints, i.e. the addition of the two lengthy migratory periods necessitates overlap between the migratory stage and the previous and subsequent stages. If we are to understand the biology of migratory species we must understand how these life-history stages interact with one another (Greenberg and Marra 2005).

Hormones mediate many of the physiological and behavioral changes as individuals transition between life-history stages (Wingfield 2008). For example, the termination of breeding and the onset of pre-basic molt is promoted by prolactin (Dawson and Sharp 1998; Dawson 2006). Numerous hormones regulate a variety of physical and behavioral traits during breeding, so it is critical to gain a complete understanding of the hormonal changes that take place as seasonally-breeding birds prepare for reproduction. Preparation for breeding in migratory songbirds may be particularly intricate because these birds are balancing the energetic and physiological constraints they experience in two
successive and particularly demanding phases of the annual cycle, migration and breeding. While it is clear that events occurring in one life history stage influence survival and reproductive success in subsequent stages (Studds et al. 2008; Tonra et al. 2011b; Paxton and Moore 2015), studies focusing on the physiological overlap between stages in free-living migrants are limited and their results are inconsistent. For example, while Tonra et al. (2013) detected increases in androgens in American Redstarts (*Setophaga ruticilla*) on the wintering grounds prior to vernal migration (Wingfield and Farner 1978a; Wingfield and Farner 1978b) and showed that testosterone increased during vernal migration in White-crowned Sparrows (*Zonotrichia leucophrys*), Bauchinger et al. (2007) did not detect any increase in migrating Garden Warblers (*Sylvia borin*).

Testosterone (T) is a steroid hormone that mediates physiology and behavior throughout the annual cycle. T plays a central role during reproduction and may be important in facilitating the transition between breeding and the stages preceding it (Ramenofsky and Wingfield 2006). In seasonally-reproducing birds, breeding preparation involves photostimulation and subsequent recrudescence of the hypothalamic-pituitary-gonadal (HPG) axis (Hahn et al. 2009). T is present in both males and females at varying levels throughout the annual cycle (Ketterson et al., 2005) and plays a primary role in the expression of breeding behaviors in both sexes (Balthazart 1983; Staub and De Beer 1997; Wingfield et al. 2001; Rosvall 2013; Goymann and Wingfield 2014). Testosterone is also a precursor to the production of estradiol in females, which is required for
ova development (Norris 1997). Since physiological breeding preparation takes approximately one month to complete (Ramenofsky 2011), this process necessarily overlaps with the previous life-history stage and testosterone levels may increase well before the breeding season begins (Tonra et al., 2013; Wingfield and Farner, 1978a, 1978b; see Wingfield et al., 1990).

In addition to its influence during the breeding season, T plays a role during vernal migration. Almost a century ago, Rowan (1925) showed that T is required for birds to develop normal migratory behaviors. Many subsequent studies have also revealed that T and/or other gonadal hormones influences the expression of various migratory traits including migratory restlessness, hyperphagia, fat deposition, and accompanying mass gains (King and Farner 1962; Morton and Mewaldt 1962; Weise 1967; Stetson and Erickson 1972; Schwabl et al. 1988b; Schwabl and Farner 1989a; Schwabl and Farner 1989b; Deviche 1995). Further, the schedule for the expression of these migratory traits is advanced when T levels are experimentally elevated (Tonra et al. 2011a; Tonra et al. 2013; Owen et al. 2014). These studies indicate that T may influence physiological aspects of migration related to energetic condition (fattening, mass gains) and given T’s influence on aggression and territoriality during the breeding season (Balthazart, 1983; Goymann and Wingfield, 2014) we might expect a link between T and resource competition during migration. While elevated T leads to increases in activity and food intake in captive birds (Wikelski et al. 1999) and to faster movement rates and more time spent foraging in free-living breeding birds
(Lynn et al. 2000), T's influence on competition during migration has yet to be explored.

This study investigates variation in T for long-distance migrants as they progress towards their breeding grounds. To do so, I measured T levels in three Nearctic-Neotropical passage migrants at a southern and a northern site during vernal migration thus representing “far” and “near” relative distances to the breeding grounds. My major hypothesis was that T increases as birds move closer to their breeding grounds during vernal migration. I tested the following specific predictions: (1) birds sampled at our northern site would have higher circulating T when compared to conspecifics sampled at our southern site; (2) males will have higher circulating T than females; (3) T and energetic condition would be positively correlated; and (4) T and the potential for competitive interactions would be correlated. In addition, I investigated the potential for an interaction between corticosterone (CORT) and T since some studies have indicated that CORT may suppress T (Deviche et al. 2001; Swett and Breuner 2008).

Materials and Methods

Study Species

The focal species of this study were three boreal-breeding Nearctic-Neotropical passerines: Swainson’s Thrush (SWTH; Catharus ustulatus), Northern Waterthrush (NOWA; Parkesia noveboracensis), and Magnolia Warbler (MAWA; Setophaga magnolia). All three winter in Central and/or South America and breed primarily in boreal regions of North America, with the southern-most
breeding in Eastern North America around 39 degrees North latitude (Poole, 2005; Figure 2). These species do not winter or breed at either of our study locations and thus are transient migrants at both.

![Breeding ranges](image)

*Figure 2. Breeding ranges for three species of Nearctic-Neotropical migrants.*

Breeding ranges for (A) Swainson’s Thrushes, (B) Northern Waterthrushes, and (C) Magnolia Warblers are provided. Sampling locations in Louisiana and Maine are indicated by filled triangles. Breeding range data were provided by BirdLife-International and NatureServe (2014).

**Capture and Sampling**

I passively captured migrants at two study locations that represent “far” and “near” relative distances to their breeding ranges (Figure 1). My southern “far” location was Johnson’s Bayou in Cameron Parish, Louisiana (29° 45’ N 93° 30’ W; hereafter “Louisiana”) where field crews operated up to 29 mist nets during April and May 2011-2014. This site is approximately two hectares in size but is located within a larger chenier forest extending approximately 20 miles along the northern coast of the Gulf of Mexico in southwest Louisiana. My northern “near” location was Appledore Island in York County, Maine (42°58’N, 70°36’W; hereafter “Maine”) where field crews operated up to 10 mist nets during May and June 2011-2014. This site is a 33-hectare island located in the Isles of Shoals archipelago and is approximately 9.5 km from the nearest point on the mainland. Nets at both locations were checked at least every 20-30 minutes.
I obtained a blood sample via brachial puncture from each individual within 10 minutes of extraction from a net (mean ± SD; 4.5 ± 2.0 min). I used either a 26- or 27-gauge needle, depending on the species, and collected blood into heparinized capillary tubes. Samples were placed on ice or in a refrigerator until centrifuged later that same day at 14,000 rpm for 10 minutes. Plasma was extracted and stored at -20°C until analyzed. Red blood cells were placed in approximately 500 μl of lysis buffer (50mM TRIS, 10mM EDTA, 1% SDS, 0.1 M NaCl) and then stored at either -20°C or 4°C. These red blood cells were used to determine the sex of individuals genetically because neither NOWA nor SWTH exhibit extensive sexual dimorphism.

Each bird was banded with a USGS aluminum leg band and measurements of wing length (unflattened wing chord; nearest 0.5 mm), cloacal diameter (nearest 0.1 mm), and body mass (nearest 0.01g) were taken. Age was determined according to Pyle (1997) as either second-year (SY) or after-second-year (ASY). Occasionally a bird was recorded as the less specific age class of after-hatching-year (AHY) if further differentiation was not possible. For MAWA, sex was determined based on plumage characteristics (Pyle 1997). Subcutaneous fat deposits were assessed to quantify energetic condition, according to Helms and Drury (1960). Sample sizes by species, location, age, and sex are provided in Table 1.

Plasma Testosterone Assays

Plasma T was determined with an enzyme immunoassay (EIA; Enzo Life Sciences Inc., #901-065; Jawor, 2007; Jawor et al., 2007). Depending on
sample volume, 20-40 μl of plasma was used and 2000 cpm of H3 labeled T (PerkinElmer) was added to each plasma sample to allow calculation of recoveries after three extractions with diethyl ether. Extracts were re-suspended in 50 μl of ethanol and 300 μl of the assay buffer provided in the EIA kit. Recoveries were determined with 100 μl from each reconstituted sample. Samples were run in duplicate with 100 μl of each reconstituted sample in the EIA. Testosterone concentrations were determined using a logistic curve (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, California) and corrected for incomplete recoveries and initial plasma volume. Intra- and inter-assay variations were determined based on three standard samples of known T concentration placed in each assay plate. Intra-assay variation ranged from 2% to 13% and inter-assay variation was 13.8%.

Table 1

Number of bird sampled in Louisiana and Maine during spring migration by age and sex

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>SY</th>
<th>ASY</th>
<th>AHY</th>
<th>SY</th>
<th>ASY</th>
<th>AHY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swainson’s Thrush</td>
<td>Louisiana</td>
<td>57</td>
<td>55</td>
<td>6</td>
<td>57</td>
<td>33</td>
<td>8</td>
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<tr>
<td></td>
<td>Maine</td>
<td>22</td>
<td>20</td>
<td>2</td>
<td>18</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>Northern Waterthrush</td>
<td>Louisiana</td>
<td>19</td>
<td>24</td>
<td>17</td>
<td>21</td>
<td>10</td>
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<td></td>
<td>Maine</td>
<td>13</td>
<td>19</td>
<td>2</td>
<td>24</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>Magnolia Warbler</td>
<td>Louisiana</td>
<td>19</td>
<td>20</td>
<td>0</td>
<td>21</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maine</td>
<td>34</td>
<td>21</td>
<td>0</td>
<td>29</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

Plasma Corticosterone Assays

Given the possibility that CORT may suppress T (Deviche et al. 2001; Swett and Breuner 2008), I determined CORT levels in a subset of our samples, dependent
on adequate plasma volume. Plasma CORT was determined with an enzyme immunoassay (EIA; Arbor Assays Inc. #K014; DeVries and Jawor, 2013). Ten μl of plasma were used with 2000 cpm of H3 labeled CORT (PerkinElmer) to allow calculation of recoveries after three extractions with diethyl ether. Extracts were re-suspended in 400 μl of the assay buffer provided in the EIA kit. Recoveries were determined with 100 μl from each reconstituted sample. Samples were run in duplicate with 50 μl of each reconstituted sample on the EIA. CORT concentrations were determined using a logistic curve (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, California) and corrected for incomplete recoveries and initial plasma volume. Intra- and inter-assay variations were determined based on four samples from a plasma pool placed in each assay plate. Intra-assay variation ranged from 6% to 9% and inter-array variation was 6%.

Genetic Sex Determination

I extracted DNA using a DNeasy Tissue Kit, #69506, Qiagen, Valencia, CA, following standard protocol from Qiagen for nucleated erythrocytes. Polymerase chain reaction was used to amplify the Chromobox-Helicase-DNA binding gene, different versions of which are found on the Z and W chromosomes of birds, using the P2 and P8 primers (Griffiths et al., 1996, 1998). PCR products were run on an agarose gel where a single band at 325 bp indicated male and two bands (one each at 325 and 375 bp) indicated female (see Appendix A).
Statistical Analyses

Prior to analyses, T and CORT data were corrected for inter-assay variation based on the standards within each assay. Further, both T and CORT failed to meet normality assumptions. Natural log (NOWA and MAWA T), square root (SWTH and NOWA CORT), or sixth root (SWTH T) transformations corrected the disparity, and thus transformed data were used in all analyses. Also, birds aged as unknown/AHY were excluded from all analyses that included age.

I calculated energetic condition for each bird by subtracting the species- and size-specific fat-free mass from the individual’s body mass, thus larger values of energetic condition are indicative of birds with larger fat stores, after correcting for size (Ellegren 1992; Owen and Moore 2006). For this calculation I used the combined long-term data sets from both study sites, Louisiana (1993-2010) and Maine (1990-2009), for each of the three species (SWTH: N = 1,877; NOWA: N = 2,265; MAWA: N = 6,011). For each species and wing chord, I regressed body mass on fat score and then used the resulting intercept in a final regression of fat-free mass on wing chord. The resulting equations solve for fat-free mass (y) where ‘x’ represents wing chord (SWTH: \( y = 0.20x + 5.8 \); NOWA: \( y = 0.09x + 7.9 \); MAWA: \( y = 0.09x + 2.6 \)).

All analyses were performed in Program R (version 3.1.2). I used linear mixed effects models to allow the inclusion of random effects (packages lme4 and lmerTest; Bates 2010; Bates et al. 2014). I selected the best model for each set of analyses using likelihood ratio tests by comparing full models (will all
possible interactions) to simpler models, which allowed detection of significant interactions and first-order effects. I evaluated significance for main effects using likelihood ratio tests that compared a model with the variable in question to the null model. If necessary, I evaluated pairwise effects using least squares means tests with Tukey correction (package lsmeans in R).

I examined T in each study species in a series of analyses. First, I investigated whether circulating T was influenced by a variety of factors measured in this study that should be considered when testing our other predictions. These factors included date (day of year), the latency between extraction from nets and blood sampling (handling time), energetic condition, and time of day (calculated as sampling time less the time of local sunrise). Analyzing these factors first allowed me to identify which variables should be included as random variables in subsequent analyses and thus in this first set analyses, age, sex, year, and location were set as random effects. Second, I analyzed whether variation in circulating T levels can be explained by age, sex, or location of sampling (far or near the breeding grounds). In this second set of analyses, year and any effects of date, handling time, time of day, and energetic condition that were supported by model selection from the first analysis were kept as separate random effects. Third, I examined whether T was correlated with two measures of competition a migrant would experience on the day of sampling. For this analysis I compared circulating T to both the total number of other migrants captured and the number of migrants captured within each species’ foraging group. MAWA, NOWA and SWTH were considered to be upper canopy, ground,
and ground/midlevel foragers respectively. All species captured at our two study locations (# species = 98, # migrants = 21,050) were broadly designated as ground/understory, midlevel/lower-canopy, and/or upper canopy foraging guilds (loosely based on De Graaf et al., 1985). Since it is likely that site-specific factors influence these measures of competition, this third set of analyses were run for each site separately and all aforementioned variables that were supported by the model selection in our first and second sets of analyses were included as random effects.

In a separate analysis, I examined the potential influence of CORT on circulating T on a subset of sampled birds. Because of their small body size I was unable to obtain enough blood from MAWA for this additional hormone analysis (mean mass ± SD; 8.2 ± 1.0 g); therefore I only investigated the potential influence of CORT in NOWA and SWTH. For these analyses, I used ANOVAs to compare CORT and T levels in both males and females sampled within 3 minutes of capture in a net (baseline CORT samples) to individuals for whom precise capture time was unknown. This design allowed me to determine whether CORT levels were higher in birds with unknown capture times and whether this influenced circulating T levels as has been indicated in some studies (Deviche et al. 2001; Swett and Breuner 2008). Finally, I analyzed the relationship between T and diameter of the cloaca using ANOVA.
Results

Testosterone Patterns Related to Sex, Location, and Age

There were no significant interactions between location, age, and sex on T levels of SWTH and none of these factors had a significant effect on T in SWTH (Table 2; Figure 3). T levels of NOWA showed no significant interactions between location, age, or sex. There was a significant effect of location on T levels in NOWA but the effects sex and age were not significant (Table 2). Post-hoc pairwise analyses revealed that male NOWA have higher T in Maine ($t = 2.6$, $p = 0.048$; Figure 3) while T in female NOWA was not different between sites ($t = 0.49$, $p = 0.96$). Further, T in male NOWA in Maine was higher than in female NOWA from both locations but only significantly so for females in Louisiana (Female Maine-Male Maine: $t = 2.5$, $p = 0.07$; Female Louisiana-Male Maine: $t = 2.7$, $p = 0.04$). I found no significant interactions between sampling location, age, or sex on T of MAWA thus first-order effects were investigated. There were significant effects of sex and sampling location on T, but not age (Table 2). While both male and female MAWA had higher T in Maine, as shown by pairwise analyses, the difference is significant in males only (Males: $t = 3.7$, $p < 0.01$; Females: $t = 1.6$, $p = 0.4$; Figure 3). Additionally female MAWA in Maine had significantly higher T than males in Louisiana ($t = 4.0$, $p < 0.01$; Figure 3).

Other Factors in Relation to Testosterone

For SWTH, none of the other variables examined (date, handling time, energetic condition, and time of day) had a significant effect on T (Table 3). There was no effect of energetic condition or time of day on T in NOWA (Table
3). In NOWA, date had a significant and positive effect on T (Table 3). Handling time also had a significant effect on T in NOWA however the 95% confidence interval for the parameter estimate overlapped zero indicating that there is no net effect of handling time. In MAWA, date had a significant positive relationship with T while the other variables tested were not related to T levels (Table 3).

Table 2

*Linear mixed model analysis of the effects of age, sex, and sampling location on testosterone*

<table>
<thead>
<tr>
<th>Species</th>
<th>Fixed Effect</th>
<th>Estimate</th>
<th>SE</th>
<th>X²</th>
<th>P</th>
<th>Comparison to Null Model</th>
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</thead>
<tbody>
<tr>
<td>Swainson’s Thrush</td>
<td>Age</td>
<td>0.89</td>
<td>0.02</td>
<td>0.76</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>0.008</td>
<td>0.015</td>
<td>0.42</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Location</td>
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<td>0.02</td>
<td>2.77</td>
<td>0.10</td>
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</tr>
<tr>
<td>Northern Waterthrush</td>
<td>Age</td>
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<td>0.14</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
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<td>0.13</td>
<td>3.22</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Location</td>
<td>-0.29</td>
<td>0.14</td>
<td>3.87</td>
<td>0.049*</td>
<td></td>
</tr>
<tr>
<td>Magnolia Warbler</td>
<td>Age</td>
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<td>0.12</td>
<td>1.81</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
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<td>0.12</td>
<td>13.16</td>
<td>0.001*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Location</td>
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<td>0.13</td>
<td>13.20</td>
<td>0.0003*</td>
<td></td>
</tr>
</tbody>
</table>

*Note. Model effect estimates and standard error is given for each species and variable. Chi-squared statistics derive from likelihood ratio tests that compared a model with the variable in question to the null model. Asterisks denote statistical significance of a variable.*

There was no effect of sex on either CORT or T levels between birds sampled within 3 minutes of capture compared to those with unknown capture times (which could have been up to 30 minutes) in either species tested (T, SWTH: $F_{1,22} = 0.37, p = 0.55$; T, NOWA: $F_{1,16} = 0.03, p = 0.86$; CORT, SWTH: $F_{1,22} = 0.34, p = 0.57$; CORT, NOWA: $F_{1,16} = 0.16, p = 0.70$). CORT was significantly higher in birds with unknown capture times compared to those
sampled within 3 minutes (SWTH: $F_{1,22} = 4.6, p = 0.04$; NOWA: $F_{1,16} = 24.6, p < 0.001$; Figure 4). However, there was no significant difference in T between birds sampled within 3 minutes of capture and those with an unknown capture time (SWTH: $F_{1,22} = 0.001, p = 0.97$; NOWA: $F_{1,16} = 2.70, p = 0.12$; Figure 4).

**Figure 3.** Circulating testosterone levels in three species sampled at a southern and a northern site during vernal migration.

*Note:* Data for male (circles) and female (triangles) Swainson’s Thrushes, Northern Waterthrushes, and Magnolia Warblers are presented. Data points are back-transformed means and error bars represent ± 1SE.

Neither the total number of migrants nor the number of migrants in each species’ foraging group was related to T at either location for any of the three species studied (Table 4). Diameter of the cloaca was not different between males and females in any of the three species studied (SWTH: $F_{1,151} = 0.39, p = 27$
Additionally, cloacal diameter was not related to circulating T levels in any three species (SWTH: $F_{1,151} = 0.55$, $p = 0.46$; NOWA: $F_{1,58} = 1.34$, $p = 0.25$; MAWA: $F_{1,60} = 0.05$, $p = 0.83$).

Table 3

*Linear mixed model analysis of the effects of energetic condition, handling time, sampling date, and time of day on testosterone*

<table>
<thead>
<tr>
<th>Species</th>
<th>Fixed Effect</th>
<th>Model Statistics</th>
<th>Comparison to Null Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Estimate</td>
<td>SE</td>
</tr>
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<td>Swainson’s Thrush</td>
<td>Energetic Condition</td>
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<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Handling Time</td>
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<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Date</td>
<td>0.0004</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time of Day</td>
<td>0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>Northern Waterthrush</td>
<td>Energetic Condition</td>
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<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Handling Time</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Date</td>
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<td></td>
<td>Time of Day</td>
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<td>Energetic Condition</td>
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<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Handling Time</td>
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</tr>
<tr>
<td></td>
<td>Date</td>
<td>0.02</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Time of Day</td>
<td>-0.008</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Note. Model effect estimates and standard error is given for each species and variable. Chi-squared statistics derive from likelihood ratio tests that compared a model with the variable in question to the null model. Asterisks denote statistical significance of a variable.*
Table 4

Linear mixed model analysis of testosterone and foraging competition.

Testosterone was compared to the total number of other migrants and to the number of migrants within each species’ foraging group (see methods)

<table>
<thead>
<tr>
<th>Species</th>
<th>Comparison</th>
<th>Louisiana</th>
<th></th>
<th></th>
<th>Maine</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$X^2$</td>
<td>$P$</td>
<td>$X^2$</td>
<td>$P$</td>
<td></td>
</tr>
<tr>
<td>Swainson’s Thrush</td>
<td>Total</td>
<td>0.26</td>
<td>0.61</td>
<td>1.52</td>
<td>0.22</td>
<td></td>
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<tr>
<td></td>
<td>Foraging Group</td>
<td>0.05</td>
<td>0.82</td>
<td>1.13</td>
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<tr>
<td>Northern Waterthrush</td>
<td>Total</td>
<td>0.33</td>
<td>0.57</td>
<td>3.30</td>
<td>0.07</td>
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</tr>
<tr>
<td></td>
<td>Foraging Group</td>
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<td>0.71</td>
<td>1.14</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Magnolia Warbler</td>
<td>Total</td>
<td>0.33</td>
<td>0.57</td>
<td>0.14</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foraging Group</td>
<td>0.37</td>
<td>0.54</td>
<td>0.35</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

Note: Statistics derive from likelihood ratio tests that compared a model with the variable in question to the null model.

Figure 4. Circulating testosterone levels and corticosterone levels for two species.

Note: Data for (A) Swainson’s Thrushes and (B) Northern Waterthrushes are presented grouped by those sampled within three minutes of capture (squares) and those in which precise capture time is unknown (circles). Data points are back-transformed means and error bars represent ± 1SE.
Discussion

Geographic Patterns of Testosterone

In all three species studied, males had higher T in Maine, where they are closer to their breeding grounds, compared to Louisiana, although this pattern was not statistically significant in SWTH. This increase in T likely coincides with testis recrudescence which has been shown to occur throughout vernal migration in other passerine species (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Bauchinger et al. 2007; Bauchinger et al. 2008). Whereas studies of captive birds also indicate that T increases as males progress through simulated vernal migration (Schwabl and Farner 1989a; Schwabl and Farner 1989b; Bluhm et al. 1991; Ramenofsky et al. 1999; Bauchinger et al. 2008), it should be noted that hormone levels between captive and free-living birds may not be comparable (see Wingfield et al., 1990).

Within songbirds, the extent of overlap between migration and breeding preparation is likely driven by the length of the migratory journey such that long-distance migrants, including Nearctic-Neotropical migrants, are expected to have substantial temporal overlap between breeding preparation and vernal migration (Ramenofsky and Wingfield 2006; Ramenofsky 2011). Field studies investigating T levels in relation to breeding ground proximity in male migrants have yielded conflicting results. Wingfield and Farner (1978a, 1978b) found that T increases during the migratory period as males approach the breeding grounds in both the long-distance subspecies (Z.l. gambelii) and the medium-distance subspecies (Z. l. pugetensis) of the White-crowned Sparrow. Bauchinger and colleagues (2007)
did not find an increase in T until Garden Warblers arrived on the breeding grounds. It should be noted that many of their samples were below their assay’s detection limit, which may have impeded the ability to detect changes in the T levels of migrating Garden Warblers. Tonra et al. (2013) also failed to find a relationship between androgen levels and distance to the breeding grounds in American Redstarts as they prepared for their vernal migration.

While androgens, including T, are generally considered to be “male” hormones, female vertebrates also have meaningful levels of circulating androgens and possess androgen receptors (Staub and De Beer 1997). In female birds, T is produced largely by the ovaries but also may be secreted by the adrenal glands, and T levels vary seasonally (Staub and De Beer 1997; Ketterson et al. 2005). In this study, I did not detect any changes in T levels in female songbirds during spring migration. Wingfield and Farner (1978a) found T to increase during migration in the long-distance migrant subspecies of White-crowned Sparrow but not in their medium distance conspecifics (Wingfield and Farner 1978a). However in both of these studies by Wingfield and Farner (1978a, 1978b), estradiol was higher in late migrants and upon arrival at the breeding grounds compared to earlier time periods. Once produced, T may be immediately converted into estradiol or may be released into general circulation for direct use or later conversion to estradiol by target tissues (Adkins-Regan 2005). While I did not measure estradiol in this study because of sampling limitations, one might predict estradiol to increase throughout the migratory period in female songbirds.
In this study I found female MAWA to have relatively high T levels at both locations. In fact, T levels in female MAWA were higher than in male MAWA at our Louisiana sampling location. Outside of the breeding season T levels in songbirds remain fairly low in both sexes, and it is apparently rare for females to have higher T than males in any season (see Ketterson et al., 2005). However, circulating levels of T in male and female Northern Cardinals (Cardinalis cardinalis) were similar during the non-breeding season (Jawor 2007; DeVries et al. 2011). Similarly, T levels in Downy Woodpecker (Picoides pubescens) females were as high as or higher than in males during the non-breeding season (Kellam et al. 2004). Both of these species are non-migratory and both sexes may defend territories outside of the breeding season. In contrast, MAWA is a long-distance Nearctic-Neotropical migrant in which males are responsible for most territory defense in the breeding season (Dunn and Hall 2010). However, other species of wood warblers are known to aggressively defend territories during the non-breeding season (Greenberg et al. 1996; Marra 2000) and MAWA have been shown to segregate by sex on their wintering grounds (Ornat and Greenberg 1990). While it is possible that female MAWA sustain high T throughout the year to promote aggressive behaviors related to winter territory defense, given the relatively low T levels seen in male MAWA early in migration and since males likely show aggressive behaviors as well, this potential explanation for female T levels in MAWA warrants more attention.

Temporal Patterns of Testosterone
In MAWA and NOWA, T increased with date, which may indicate that the pattern detected between sampling locations is a function of the time since breeding preparation began, rather than the geographic proximity to the breeding grounds. This observed pattern makes sense because after being photostimulated, the HPG axis increases production of hormones including T (Deviche and Small 2001; Hahn et al. 2009). Vernal migration takes approximately 20 days for long-distance migrants complete (Wikelski et al. 2003; Stutchbury et al. 2009; Ewert et al. 2012). Based on my mixed-effect models, I estimate that circulating T would increase by 1.3 ng/ml in both NOWA and MAWA during that time. Testosterone levels in breeding male songbirds are generally within the 1 – 5 ng/ml range (Ketterson et al. 2005), so this observed within-season increase represents a meaningful change in hormone levels.

Other Factors Considered in Relation to Testosterone

Testosterone was not related to time of day, energetic condition, cloacal protuberance, or handling time. Previous studies have found that T follows a diel rhythm with highest levels during the over-night or early morning periods (Hau et al. 2002; Kempenaers et al. 2008; Goymann and Trappschuh 2011). Given that I only sampled T during daytime hours, it is not surprising that I did not find T to correlate with time since sunrise. Removal of T has been shown to suppress the development of migratory traits including fattening and increases in mass (Stetson and Erickson 1972; Schwabl and Farner 1989a; Deviche 1995) and experimental increases in T have been shown to promote the development of such traits (Owen et al. 2014; Tonra et al. 2011b; 2013). Although understudied,
T may also play a role in the regulation of muscle anabolism (see Ramenofsky, 2011) and these seemingly opposing roles of T with regard to condition may explain the lack of relationship in this study. While some studies have indicated T levels may be related to condition changes in preparation for migration (Tonra et al. 2011b; 2013) it is likely that T’s role in fattening and other migratory traits is complicated and requires further study (Ramenofsky and Németh 2014).

The cloacal region is used for sperm storage in male birds and studies have shown that T plays a role in the development of the cloacal protuberance (Tonra et al. 2011a; Ramenofsky and Németh 2014). Given that the proportions of migrating songbirds that have been found to produce measurable amounts of sperm is low (Quay 1985a; Quay 1985b; Quay 1986) and that T levels I report in this study are lower than what is typical of breeding songbirds (Ketterson et al. 2005), the lack of relationship between cloacal diameter and T is not unexpected. That said, Tonra et al. (2011a) found an inverse relationship between cloacal diameter and arrival date on the breeding grounds with early arriving males having larger cloacal diameters, higher androgen levels, and better breeding success. In my study, cloacal diameter was not different between males and females indicating that that development of the cloacal protuberance in males had yet to commence.

Time spent in captivity has the potential to influence hormone levels and studies often exercise caution by including handling time in statistical analyses of T and other hormones. However, the effect of relatively short handling times (< 30 min), as in this study, are not likely to significantly influence circulating T
(Peters et al., 2001; but see Devries et al., 2011). While I define handling time as the latency between extraction from a net and sampling, precise capture time in a net is unknown for most of the birds in this study. Even though previous studies have suggested that high levels of CORT caused by capture and handling may suppress T and other hormones of the HPG axis (Deviche et al. 2001; Swett and Breuner 2008), I saw no indication of this in my study. Testosterone levels in birds with elevated “stress” levels or CORT were no different from birds with lower, baseline CORT levels. My results are not novel, however, as other studies have found similar results (e.g. Devries et al., 2011; Wikelski et al., 1999), but this study does add to a growing body of evidence of the complex relationship between these two hormones. For example, higher CORT levels may increase free (unbound) T (Deviche et al. 2001) and variation among levels of T, CORT, and their shared binding globulin do not seem to influence T’s availability to target tissues (Swett and Breuner 2008).

Testosterone is known to mediate inter- and intra-specific aggression and territorial defense in songbirds during the breeding season (Balthazart 1983; Wingfield et al. 2001). In this study, however, I found no evidence that T was related to or influenced by competition during migratory stopover as assessed by both the total number of other migrants present and the number of migrants within a species’ foraging group. That said, T may influence the rate of and time spent foraging during the breeding season (Lynn et al. 2000) and thus the relationship between T, competition, and foraging activities during migration warrants further study during the migratory periods.
Conclusions

The growing consensus is that male migrants increase their T during vernal migration, likely as a result of overlapping life-history stages as breeding preparation begins. While female migrants did not vary during migration in this study, most birds of both sexes had slightly higher T levels than what is found in other Nearctic-Neotropical migrants during the wintering period (e.g. Tonra et al., 2013; Wingfield and Farner, 1978a, 1978b). Additionally, my observed temporal increase in T during migration may reflect the time that has elapsed since photostimulation rather than simply a geographic proximity to the breeding grounds. The overlapping life history stages of breeding and migration seen in this and other studies likely facilitate a smooth transition to commence breeding activities (e.g. territory establishment) immediately upon arrival on the breeding grounds.
CHAPTER III – OVERLAPPING LIFE-HISTORY STAGES IN MIGRATING SONGBIRDS: VARIATION IN CIRCULATING TESTOSTERONE AND TESTOSTERONE PRODUCTION CAPACITY

Covino KM, Jawor JM, Kelly JF, Moore FR (in Revision) Overlapping life-history stages in migrating songbirds: Variation in circulating testosterone and testosterone production capacity. J. Ornithol

Abstract

Understanding the extent of overlap between life-history stages is fundamental to understanding full-life cycle biology especially for migratory species. Testosterone levels vary throughout the annual cycle in seasonally reproducing vertebrates. In migratory songbirds, testosterone increases associated with breeding preparation may overlap with the vernal migratory period; however, this overlap remains largely unexplored. I test the hypothesis that both circulating testosterone and the capacity to elevate testosterone increases throughout vernal migration in long-distance songbird migrants. Using stable hydrogen isotopes, I relate testosterone in songbirds sampled en route to breeding ground proximity as determined by the stable hydrogen isotope ratio in their feathers. I determined capacity to elevate testosterone using field gonadotropin-releasing hormone bioassays. Males that were closer to their breeding grounds had higher circulating testosterone; whereas there was no relationship between testosterone and breeding ground proximity in females. Similarly, while capacity to elevate testosterone was not related to breeding ground proximity in female migrants, this capacity was greater in males closer to
their breeding grounds than those further away from their breeding grounds. These results reveal that male migrants prepare for breeding during their vernal migration, whereas the schedule for breeding preparation among females is less clear and may be more complex.

Introduction

Seasonally reproducing animals adjust their behavior, physiology, and morphology as they progress through the annual cycle (Jacobs and Wingfield 2000; Piersma and van Gils 2011). The transition from one stage of the annual cycle to the next often results in overlap between stages, especially in migratory species that must balance the demands of migration with those of the subsequent stage (Ramenofsky 2011). For example, during vernal migration songbirds must not only devote time and energy towards completing their journey, but they must also begin the development of breeding characteristics or they risk a mistimed breeding attempt. Understanding the amount and implications of overlap between vernal migration and the development of breeding is a crucial step toward understanding the full-life-cycle biology of migratory species (Marra et al. 2015). The extent of overlap between breeding preparation and vernal migration in long-distance migrating songbirds, however, is largely unexplored (but see (Bauchinger et al. 2007; Tonra et al. 2013; Covino et al. 2015). Previous research into this overlap has focused on two related aspects of breeding preparation: (1) changes in breeding hormone levels (e.g. testosterone), and (2) changes in gonad size and/or developmental state.
Testosterone (T) is an influential hormone in birds and other vertebrates (Adkins-Regan 2005) that mediates many aspects of physiology and behavior (Wingfield et al. 2001). Testosterone’s wide-ranging influences include gonad recrudescence, gamete development, and development of mating behaviors and are central to the breeding biology of seasonally reproducing birds (Ramenofsky and Wingfield 2006). Previous field studies investigating changes in circulating T during migration have sampled birds at different locations across their wintering, migratory, and/or breeding range (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Bauchinger et al. 2007; Covino et al. 2015). These studies attribute between-site variation in circulating T to relative proximity to the breeding grounds. This study design largely ignores variation in circulating T among individuals within a site, which may be related to individual variation in proximity to breeding destination. Further, studies have reported inconsistent findings among species: In some species, T increased throughout vernal migration in both sexes (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Schwabl and Farner 1989a; Schwabl and Farner 1989b); in others, only males increase their T levels during vernal migration (Covino et al. 2015); and in still others, T remained the same during migration in both males and females (Covino et al. 2015). Even studies that sampled only male songbirds report conflicting results–some found an increase in circulating T with decreasing distance to the breeding range (Bluhm et al. 1991; Ramenofsky et al. 1999) and others failed to detect a change (Bauchinger et al. 2007; Tonra et al. 2011a; Owen et al. 2014).
The capacity to elevate T is also likely to reflect reproductive status (Wingfield et al. 1979; Jawor et al. 2006; DeVries et al. 2011). Each spring the hypothalamic-pituitary-gonadal (HPG) axis hormonal pathway is naturally upregulated in both male and female songbirds when nuclei in the hypothalamus are photostimulated by increasing day length (Adkins-Regan 2005). The subsequent increased production of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the production of the gonadotrophic hormones (Adkins-Regan 2005). Luteinizing hormone (LH) promotes T production in testicular Leydig (males) and in ovarian thecal cells (females). In females, follicle stimulating hormone (FSH) promotes aromatase activity, which facilitates conversion of some T to estradiol, while in males FSH promotes spermatogenesis by the Sertoli cells and growth of the seminiferous tubules (increased testis size; Adkins-Regan 2005). It should be noted that both T and estradiol mediate breeding behaviors and physiological changes in females. Additionally female vertebrates, including birds, possess “physiologically meaningful levels of androgens” (Staub and De Beer 1997). GnRH bioassays may be performed by injecting individuals with a large dose of GnRH, thus stimulating the HPG axis. Testosterone levels following treatment with GnRH are indicative of the maximum capacity to elevate T (Jawor et al. 2006) and can measure HPG axis activity and preparation for breeding in both sexes (Schoech et al. 1996).

In this study I employ stable hydrogen isotopes to geographically link individual Black-and-white Warblers (*Mniotilta varia*) captured during vernal
migration to breeding area destinations. I then used these links to investigate the impact of individual variation in proximity to breeding location on both circulating T and capacity to produce T in this long-distance migrant. This unique approach enables me to study migrating individuals from across the entire breeding range of a species while they pass through a single study site. I hypothesized that circulating T will be related to breeding destination in both males and females, with individuals closer to their breeding grounds having higher levels of circulating T. I further hypothesized that the capacity to produce T will show a similar relationship with proximity to breeding location such that response to an injection with GnRH would be higher in individuals closer to their breeding grounds.

Methods

Field and Laboratory Data Collection

My study was conducted in Louisiana (USA) along the northern Gulf of Mexico coast (29°45'N 93°30'W; Figure 5). I captured Black-and-white Warblers (*Mniotilta varia*) with mist-nets operated daily during spring migration from 18-Mar to 12-May, 2012-2014. The Black-and-white Warbler is a Nearctic-Neotropical migrant with its breeding range extending from the southeastern U.S. throughout eastern North America and central Canada (Kricher 2014; Fig 1). Within 10 min (Mean ± S.D.: 4.9 ± 2.1) of extraction from a net I obtained a blood sample (approximately 60 μl) via the brachial vein. Blood was collected into a heparinized capillary tube, kept on ice until centrifuged, and plasma was then drawn off and stored at -20°C until analyzed. To quantify T production capacity I
employed GnRH field bioassays which cause the pituitary to release LH and tests the gonads to see if they have a higher capacity to produce T than what is being generally released into circulation (typically considered ‘baseline’ levels; Jawor et al. 2006; Busch et al. 2008). Black-and-white Warblers were given a 50 μl injection of GnRH solution made from 2.5 μg of chicken GnRH-I (American Peptide Co., #54-8-23) dissolved in 1 M phosphate-buffered saline (PBS). This dosage of GnRH has previously been shown to be sufficient to stimulate maximal T production in similarly sized birds (Jawor et al. 2006). Injection sites were cleaned with a sterile alcohol swab and injections were given intramuscularly into the pectoralis major using a 25 gauge needle connected to a Hamilton® syringe with a Luer tip (Hamilton Laboratory Products, Part #80601; Jawor et al. 2007). Results of a small study (N = 40) of Black-and-white Warblers sampled at various time-points post-injection indicated that peak in response occurs after a 30 min holding period (see time trials in Results). Therefore all other study subjects were held in a cloth bag for 30 min after which a post-injection blood sample was obtained. Throughout the study, a random subset of birds were injected with PBS only instead of GnRH as controls since PBS injections do not stimulate gonadal T production (Jawor et al. 2006; DeVries et al. 2011). Birds were then banded with a USGS leg band, the left and right fifth rectrices were pulled for later hydrogen isotope analysis, and measurements of wing chord and body mass were taken. Sex was determined based on plumage characteristics and age was classified as either second-year (SY) or after-second-year (ASY) based on feather wear and/or molt limits (Pyle 1997).
Since corticosterone (CORT) may suppress T (Deviche et al. 2001; Swett and Breuner 2008) I determined CORT levels in a subset of individuals. Since CORT increases upon perception of a “stressor” which includes capture (Romero and Reed 2005), I sampled birds within 3 minutes of capture (baseline CORT samples) and compared their CORT levels to individuals for whom precise capture time was unknown. I presumed that birds with an unknown capture time were in a net longer than 3 minutes and thus would have higher levels of CORT than birds sampled within 3 minutes (Angelier et al. 2010). This design allowed me to determine whether birds with higher CORT levels differed in circulating T levels from birds with lower baseline CORT.

![Map of the breeding range of the Black-and-white Warbler (Mniotilta varia).](image)

*Figure 5. The breeding range of the Black-and-white Warbler (Mniotilta varia).*

Note: The breeding range of the Black-and-white Warbler (Mniotilta varia) extends across eastern North America and western Canada. The sampling location at Johnson's Bayou, Louisiana is indicated. Map generated using data from BirdLife International (BirdLife-International and NatureServe 2014).
As described in Jawor et al. (2007) and Covino et al. (2015), I determined plasma hormone levels using enzyme immunoassays (T: Enzo Life Sciences Inc., #901-065 and CORT: Arbor Assays Inc., #K014). Briefly, 2000 cpm of tritium labeled hormone (PerkinElmer) was added to 20-40 μl (T assay) or 10 μl (CORT assay) of plasma from each subject and repeated samples from the same subject were analyzed on the same assay plate. I calculated recoveries after three extractions with diethyl ether and re-suspending each extract with 50 μl ethanol (T assay only) and 300 μl (T assay) or 400 μl (CORT assay) of the buffer provided in the assay kit. Mean recoveries for T assays were 83% and 79% for CORT assays. I used a logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories) to calculate hormone levels and corrected for incomplete recoveries and initial plasma volume. For T, intra-assay (0.65% to 11%) and inter-assay (11.7%) variation were determined using three standards in each assay and data were corrected for inter-assay variation before analysis. CORT samples were run in a single assay and intra-assay variation was 5.6% based on four standards.

Black-and-white Warblers complete their prebasic molt on their breeding grounds where all feathers (in adults) or body feathers (in young of the year) are replaced (Pyle 1997). Because young birds grow their flight feathers in the nest, the tail feathers of both the adults (ASY in spring) and young (SY in spring) carry the hydrogen isotope signal from the breeding ground until they complete their next prebasic molt. Feathers were prepared for stable hydrogen isotope analysis at the University of Oklahoma. Feathers were cleaned by immersing them in 2:1
chloroform:methanol for 30 s (Paritte and Kelly 2009). Each sample was allowed to air dry for 24 h under a fume hood. Samples were cleaned in detergent once with 1 L of a 1:30 (detergent: deionized water; v/v) solution of Fisher Versa-Clean (Fisher Scientific, #04-0342), then rinsed the samples three times in three 1 L baths of deionized water, and allowed the samples to air dry for 24 h under a fume hood. Tissue samples were weighed and 350 ug ± 10 of each were loaded into 3.5 mm × 5 mm silver capsules. I ran duplicate samples and standards comprised of replicates of an in-house standard (finely powdered feathers from a brown-headed cowbird; Kelly et al. 2009) and USGS-40 and one USGS-41 purchased from the National Institute of Standards and Technologies. Samples were analyzed to determine hydrogen isotope-ratios at the Colorado Plateau Stable Isotope Facility and data were corrected for instrumental drift within runs. Data are presented using standard delta notation (δ²H) calculated as [(isotope ratio sample/isotope ratio standard) − 1] × 1000 for the ratio of heavy (²H) to light (¹H) isotopes in each sample. Thus data presented as parts per thousand (‰) with higher values representing relatively more of the heavier isotope.

Data Analysis

All T and CORT data were corrected for inter-assay variation based on the standards placed within each assay plate. Response to the GnRH injection was calculated as the difference between initial (pre-injection) and post-injection levels of T. Thus negative response values and those close to zero indicate a lack of response while positive values indicate a greater capacity to produce T relative to circulating T levels. Testosterone, response to GnRH, and CORT data
were transformed with cubed root (T and response to GnRH) and log (CORT) functions to achieve normal distributions (Shapiro-Wilks test). I estimated the energetic condition of each individual by first determining the size-specific fat free mass from a large dataset of Black-and-white Warblers (N = 1,145) and subtracting that value from the bird’s body mass (Owen and Moore 2006).

All analyses were performed in Program R (version 3.1.3). The effect of control injections (PBS) were evaluated using a repeated measures (within subjects) ANOVA on T with Sex and Sample (pre- or post-injection) as fixed factors. I also used ANOVA to compare CORT and T in birds with known capture times (within 3 minutes of capture) to individuals whose precise capture time was unknown.

I performed a series of analyses using linear mixed models with package lme4 (Bates 2010; Bates et al. 2014). For each analysis, I first evaluated the potential for interactions among fixed effects by comparing models with all possible interactions to simpler models (with sequentially fewer interactions). Significance of each variable was then determined with a nested model comparison approach by comparing the full model to the model without the variable in question. All model comparisons were evaluated using likelihood ratio tests with the Chi-squared distribution.

Using the approach outlined above, I performed the following analyses: (1) A time-series analysis with sex and sample (0, 15, 30, 45 min.) as predictor variables, individual as a random effect, and T as the response variable. Here I also evaluated pairwise effects across samples using least squares means tests.
with Tukey correction using the R package lsmeans. (2) An evaluation of additional factors that may influence T with date (ordinal day of year), handling time (the latency between extraction from nets and blood sampling), energetic condition, and time of day (calculated as sampling time less the time of local sunrise) as predictor variables. Here, circulating T was the response variable and feather hydrogen, age, sex, and year were held as random variables. This approach allowed me to identify which factors may influence T independent of my focal variables (e.g. sex, feather hydrogen). (3) An analysis of circulating T with feather hydrogen, age, and sex as predictor variables and T as the response variable. In this analysis time of day and year were held as random variables. (4) An analysis of response to the GnRH injection using feather hydrogen, age, and sex as predictor variables, GnRH response (post-injection T less pre-injection T) as the response variable, and time of day and year included as random variables. (5) Feather hydrogen analysis with date and sex as predictor variables, feather hydrogen as the response variable, and year as a random variable.

Results

**Time Trials and Control Injections**

There was a significant effect of the time at which the post-injection sample was taken on the post-injection T level ($\chi^2_{(3)} = 19.48, P < 0.001$). Paired tests revealed that in both males and females, samples taken 15 or 45 min post-injection had similar T levels to initial samples ($P > 0.10$ for each comparison). But samples taken at 30 min had significantly higher T levels than initial samples ($P < 0.01$; Figure 6). When analyzing samples from control birds, I found no
interaction between sample (pre- or post-injection) and sex ($F_{1,14} = 0.2$, $P = 0.6$) and no difference between samples ($F_{1,14} = 0.03$, $P = 0.9$).

**Figure 6.** Time trials for GnRH injections in Black-and-white Warblers.

Note: Testosterone levels in migrating male and female Black-and-white Warblers prior to an injection with GnRH (Time = 0) and at various time points post-injection. Data points represent group means and error bars represent standard error. Sample sizes within each time period are provided. Results indicate that the maximum response to the GnRH injection occurs 30 minutes after the injection.

**Circulating Testosterone**

I did not find any influence of energetic condition, handling time, or day of year on circulating T when feather hydrogen was held as a random variable (Table 5). However, time since sunrise had a significant and negative influence on circulating T (Table 5) and thus was held as a random variable for all subsequent analyses. I detected a significant 3-way interaction when evaluating
the relationships among age, sex, and feather hydrogen and circulating T ($\chi^2(4) = 13.0, P = 0.01$); therefore I analyzed males and females separately. In males, feather hydrogen was strongly related to T such that individuals with more depleted feather hydrogen levels, indicating a more northern breeding destination, had lower circulating T (Table 5; Figure 7A). Additionally, older birds (ASY) had higher T than younger (SY) birds (Table 5). In females, neither feather hydrogen nor age was related to circulating T (Table 5; Figure 7B).

Figure 7. Patterns of testosterone relative to feather hydrogen levels in migrating Black-and-white Warblers.

Note: Feather hydrogen levels represent breeding destination. Data are from male (A) and female (B) Black-and-white Warblers during their spring migration. Trend line indicates overall linear pattern and shaded areas are 95% confidence intervals. More depleted (negative) feather hydrogen ratios indicate northern breeding grounds and less depleted ratios indicate southern breeding grounds.
Table 5

*Linear mixed model analyses of circulating testosterone, response to the GnRH injection, and feather hydrogen levels in Black-and-white Warblers*

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<th>N</th>
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<th>Estimate</th>
<th>SE</th>
<th>X²</th>
<th>P</th>
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Note: Fixed effects that were evaluated for each response variable are listed. Chi-squared statistics derive from likelihood ratio tests that compared the full model to a model without the variable in question. Asterisks denote statistical significance of a variable. Sample sizes for each analysis are provided.
Corticosterone levels were determined in a subset of individuals (N = 21) due to the need for adequate plasma volume for both hormone assays. I compared circulating T and CORT in birds sampled for baseline CORT (within 3 minutes of capture) to individuals for whom exact capture time was unknown. CORT was significantly elevated in birds with an unknown capture time compared to those sampled within 3 minutes of capture ($F_{1,19} = 8.7, P < 0.01$). However, T levels between these two groups were similar ($F_{1,19} = 0.5, P = 0.5$). Given these results I do not believe my unknown capture times or variation in CORT influenced the results of this study.

**Testosterone Response after GnRH Injection**

There was a significant interaction between feather hydrogen and sex on GnRH response ($\chi^2_{(1)} = 6.1, P = 0.01$) and thus males and females were analyzed separately. Males that had less depleted feather hydrogen levels, indicating a more southerly breeding destination, showed a greater response to the GnRH injection (Table 5; Figure 8A). While many females had a strong response to the injection, some showed no response, and there was no relationship between GnRH response and feather hydrogen values and thus no indication this is related to migration distance in females (Table 5; Fig 8B). There was no difference in the GnRH response between the age groups in either sex (Table 5).

**Variation in Feather Hydrogen**

Both date and sex had significant effects on feather hydrogen (Table 5). Males had more depleted feather hydrogen values compared to females.
indicating that a higher proportion of males passing through my site are heading to northern breeding grounds and a higher proportion of females are heading to breeding grounds that are more southerly. Overall, feather hydrogen decreased with date such that birds passing through my site earlier in the season had feather isotope signatures representative of southeastern breeding grounds and those passing through later in the season had feather isotope signatures indicative of boreal breeding grounds (Figure 9). I found no difference in the timing of males and females when I controlled for feather hydrogen ($\chi^2(1) = 2.6$, $P = 0.1$).

Figure 8. Testosterone response to gonadotropin-releasing hormone bioassays in migrating Black-and-white Warblers in relation to feather hydrogen isotope ratios.

Note: Data from male (A) and female (B) migrants are presented. Dotted lines indicate the best fit relationship and shaded areas are 95% confidence intervals. Positive responses indicate that post-injection testosterone levels were higher than pre-injection levels and represent birds in a more advanced stage of breeding preparation. More depleted (negative) feather hydrogen ratios indicate northern breeding grounds and less depleted ratios indicate southern breeding grounds.
Figure 9. Pattern of feather hydrogen values relative to passage date in Black-and-white Warblers during spring migration.

Note: Data from male (filled circles) and female (filled triangles) are presented. More depleted (negative) feather hydrogen ratios indicate northern breeding grounds and less depleted ratios indicate southern breeding grounds.

Discussion

This study tests whether circulating T and capacity to produce T vary with proximity to breeding destination in migrating songbirds. Male Black-and-white Warblers sampled at the same site, and experiencing similar environmental conditions, are at different states of reproductive preparation. Males closer to their breeding grounds had higher T levels and a higher production capacity. Testosterone levels observed in males closer to their breeding grounds were similar to levels seen in males of another parulid (wood warbler) species upon arrival on the breeding grounds (Tonra et al. 2011b). My results are similar to studies that have compared T levels in birds sampled at different locations throughout spring migration (Wingfield and Farner 1978a; Wingfield and Farner
Previous studies have also indicated that testis size increases throughout vernal migration (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Bluhm et al. 1991; Bauchinger et al. 2005; Bauchinger et al. 2007), which points to an increase in the gametogenic ability of males as they travel towards the breeding grounds.

Older males that had already completed at least one breeding season had significantly higher circulating T than those that had yet to complete their first breeding season. Similarly, Tonra et al. (2013) found that circulating androgens (collectively T and other related hormones) in older male songbirds increased prior to departure on vernal migration from the wintering grounds, but this was not true of younger individuals. Previous studies on White-crowned Sparrows (Zonotrichia leucophrys orianthi) have also demonstrated age class differences in breeding physiology in both circulating T levels (Morton 2002) and testis size during breeding (Morton et al. 1990). These differences indicate that the development of the HPG axis in younger males may be delayed relative to older males (see Morton 2002). One explanation for this result could be that, since they are undertaking their first spring migration, younger birds may be less able to predict how close they are to their breeding destination. Alternatively, young birds may winter in lower quality habitats compared to adults (Marra 2000) and this may affect the onset and rate of breeding development.

Although the schedule for gonad maturation is less well known for females, studies have indicated that ovarian development may begin before and continues during vernal migration (Wingfield and Farner 1978a; Wingfield and
Farner 1978b; Schoech et al. 1996; Wingfield et al. 1997). However, similar to Covino et al. (2015), female Black-and-white Warblers in this study lacked a relationship between circulating T or T production capacity and breeding ground proximity; but this does not rule out change in other hormones important for reproduction in females. Wingfield and Farner (1978a; 1978b) found that T increased during spring migration in females of the long-distance subspecies of the White-crowned Sparrow (Z. l. gambelii) but not in their short-distance conspecifics (Z. l. pugetensis). Interestingly, estradiol, which is produced via a conversion from T, did increase in both subspecies during spring migration in these same studies. Estradiol is required for ova development (Norris 1997) however, T may be converted into estradiol immediately, in the ovary, or it may be converted before use by target tissues (Adkins-Regan 2005). Although sampling constraints prevented me from measuring estradiol in this study, variation in estradiol levels in addition to circulating T should be evaluated in future studies of breeding preparation phenology in female migrants. As suggested in some studies, final maturation of ovarian follicles may depend on environmental or social cues on the breeding grounds such as the presence of a territorial male (King et al. 1966; Farner and Lewis 1971; Ball and Ketterson 2008) and this may explain the lack of relationship between measures of reproductive readiness and breeding ground proximity in female migrants.

In my study, precise capture time was unknown for most individuals; capture and handling is perceived as a stressor in birds. Differential impacts of CORT on T levels by sex might contribute to the differences I found between the
sexes. Although handling time and the subsequent increase in CORT have the potential to interfere with T production (Deviche et al. 2001; Swett and Breuner 2008), I saw no indication of this effect in my study for either sex. Similar to Wikelski et al. (1999) and DeVries et al. (2011), I found that individuals with elevated “stress” levels of CORT and individuals with lower baseline CORT levels had similar T levels. Therefore it is unlikely that the higher CORT levels experienced by some birds in my study influenced my findings.

In this study migrants destined to breed in the southern part of the breeding range passed through my study site earlier than northern breeders, which is consistent with a previous study on the same species at the same study site (Paxton and Moore 2015). Paxton and Moore (2015) also demonstrated that the timing of passage was influenced by the quality of wintering habitat from which a bird was coming regardless of breeding destination. In my study, date (day of year) was not directly related to circulating T in Black-and-white Warblers. However, Tonra et al. (2013) found that circulating androgens not only increased in male songbirds prior to departure from the wintering grounds, but also that this increase was correlated with energetic condition. Circulating T and energetic condition were not related in this study nor was condition related to date in Paxton and Moore’s (2015) study of the same species. It is possible that while wintering habitat quality influences condition and departure date from the wintering grounds (e.g. Studds et al. 2008) and passage date during migration (Paxton and Moore 2015), these effects do not translate into differences in breeding preparation phenology during migration. That said, some studies have
indicated that early arriving males on the breeding grounds are at a more advanced stage of breeding readiness (e.g. Tonra et al. 2011b). Similarly, male Black-and-white Warblers heading to more southerly breeding grounds passed through my study site earlier than their northern breeding counterparts and these earlier migrants had higher circulating T levels. This finding lends support to early arriving males being in a more advanced stage of breeding readiness. However, I found that when feather hydrogen, as a proxy for breeding destination, is controlled, there was no relationship between circulating T and date of passage. Thus earlier males have higher circulating T due to their close proximity to the breeding grounds rather than due to their earlier passage.

Most studies of T during vernal migration have found that male songbirds increase circulating levels as they proceed towards the breeding grounds. Studies of female migrants are few and while some have detected increases in T during migration, others have not. Capacity to produce T has not been well studied during migration, and here I demonstrate that capacity was strongly related to breeding ground proximity in male, but not female Black-and-white Warblers. This pattern of increasing capacity throughout migration in males mirrors increases in their circulating T levels. As previous studies have shown, the relative distance to the breeding grounds is related to the degree to which migrating songbirds are physiologically prepared for breeding. This overlap between migration and breeding preparation is necessary to ensure adequate breeding preparation prior to arrival on the breeding grounds.
CHAPTER IV – SEX-SPECIFIC HYPOTHALAMIC-PITUITARY-GONADAL AXIS ACTIVITY IN MIGRATING SONGBIRDS

Abstract

In seasonally migratory species, the overlap between the migratory and breeding life history stages is a balance between the physiological and behavioral requirements of each stage. Previous studies investigating the degree to which songbirds prepare for breeding during spring migration have focused on either circulating hormone levels or direct measures of gonadal recrudescence. In this study, I evaluated breeding preparation in long-distance songbird migrants by assessing hypothalamic-pituitary-gonadal (HPG) axis activity with gonadotropin releasing hormone (GnRH) bioassays throughout the migratory period. During spring migration both males and females had a significant response to GnRH injections as reflected in elevated testosterone levels. The magnitude of response to GnRH injections, $R_{potential}$, in females stayed consistent throughout spring migration; however, $R_{potential}$ in males increased as the migratory season progressed. It is clear that at least some amount of breeding preparation occurs either before or during spring migration in both sexes, however the schedule of preparation appears to be sex-specific. Males and females may employ different strategies for the development of the breeding stages. While increasing day length initiates development in both sexes, in males this development continues at a steady pace throughout the migratory period. Females, however, may rely more on supplementary or local cues from the breeding grounds thus delaying the final stages of follicular development until
after arrival. These sex-specific differences in the strategy for the development of breeding warrant future investigations for both male and female songbirds. Moreover, research focused on how the development of breeding is balanced with the expression of migratory traits in long-distance songbird migrants is needed.

Introduction

Testosterone (T) is a steroid hormone that mediates a wide-range of behavioral and physiological effects in songbirds and other vertebrates (Wingfield and Silverin, 2009; reviewed in Adkins-Regan 2005). While T is linked to many actions throughout the annual cycle, it has been primarily studied in the context of the breeding season (e.g. Ramenofsky and Wingfield, 2006; but see Ramenofsky et al., 1992, 1999; Schwabl et al., 1988). Many of the effects mediated by T are central to the expression of male breeding behaviors, including singing and aggressive territory defense (Wingfield et al. 2001; Adkins-Regan 2005; Goymann and Wingfield 2014). Testosterone is also necessary for the expression of female breeding behaviors either acting directly on androgen receptors or following conversion to estradiol and by interacting with estrogen receptors (Norris 1997; Staub and De Beer 1997; Ketterson et al. 2005; Rosvall 2013). Typically peak levels of circulating T occur during the early breeding season in male songbirds, and several studies have focused on circulating levels of T throughout the annual cycle in order to investigate breeding preparation and hypothalamic-pituitary-gonadal (HPG) axis activity (e.g (Wingfield 1984; Jawor et al. 2006); see Ketterson et al. 2005). While only a few studies have investigated
T variation in female songbirds, detectable levels are generally found throughout the annual cycle (e.g. Jawor et al., 2007; see Ketterson et al., 2005).

The coordination of physiological and behavioral traits associated with each stage of the annual cycle must allow adequate time and energy for development and expression of those traits (Jacobs and Wingfield 2000; Ramenofsky 2011). In addition to the stages expressed by all birds—breeding, molt, non-breeding, migratory birds face the challenge of also expressing spring and fall migration stages and, in some species, an additional molt. This increase in the number of stages in the annual cycle results in less flexibility in the timing of each stage and more overlap between stages (Ramenofsky 2011). In long-distance migrants, especially those breeding at northern latitudes, the opportunity to breed is limited to a relatively narrow time-frame; thus proper timing of the expression of breeding traits upon arrival is crucial (Jacobs and Wingfield 2000; Ramenofsky and Wingfield 2006). Estimates suggest that it takes approximately one month for songbirds to fully activate the HPG axis and to complete gonadal recrudescence in preparation for the breeding season (Ramenofsky 2011). Given the time constraints restricting long-distance migrants upon arrival on the breeding grounds, HPG axis activity should increase before or during spring migration. Males should be prepared to quickly transition to expressing breeding behaviors once on the breeding grounds by establishing and defending territory and attracting mates if environmental conditions are conducive. Similarly, if environmental conditions are appropriate for breeding, females should arrive on the breeding grounds receptive to courting males since the initiation of breeding
early yields greater breeding success (Smith and Moore 2005; Wingfield and Ramenofsky 2011). Thus arriving individuals must have a sufficiently developed HPG axis to promote the expression of these behaviors and to facilitate a quick transition to the breeding life-history stage. While some male songbirds have detectable increases in T during their migration towards the breeding grounds (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Covino et al. 2015) this pattern is not universal (Bauchinger et al. 2007; Covino et al. 2015). Studies of breeding hormone fluctuations in female migrants are relatively rare but indicate that T levels may not increase until after females reach the breeding grounds (Covino et al., 2015; Wingfield and Farner, 1978a, 1978b; Chapter III).

Several studies have noted links between preparation for breeding and expression of migratory characteristics (Tonra et al. 2011a; Ramenofsky and Németh 2014; Owen et al. 2014). That said some of the behavioral and physiological effects linked to high T levels may be antagonistic to the expression of the migratory syndrome (Ketterson et al. 2015) and a successful migratory journey. For example, T mediated territoriality while en route may be costly when a migrant is time constrained, which is especially likely during spring passage. High concentrations of T may negatively impact lipogenesis and fuel deposition rates potentially interrupting migratory behavior (Ketterson et al. 1991; Deviche 1995). In contrast, several studies have indicated that the expression of various migratory traits is linked to increasing T levels (Rowan 1925; Rowan 1929; Weise 1967; Deviche 1995). For example, the expression of migratory characteristics including fattening, mass gains, and migratory restlessness, may be dampened
or delayed when T is absent (Lofts and Marshall 1961; King and Farner 1962; Morton and Mewaldt 1962; Stetson and Erickson 1972; Schwabl et al. 1988b; Schwabl and Farner 1989a). Further, the administration of T induces the expression of migratory behaviors in captive birds (Tonra et al. 2011a; Tonra et al. 2013; Owen et al. 2014).

To facilitate a timely transition between migration and breeding life history stages while avoiding the antagonistic effects of prolonged T elevation during migration itself, it is likely that increases in HPG axis activity outpace changes in circulating hormone levels (Chapter III). Further, while levels of circulating T or other breeding hormones (luteinizing hormone, estradiol, dihydrotestosterone) may reflect reproductive condition, individuals may express short-term fluctuations in hormone levels for many reasons (Wingfield et al. 1990). Therefore, these fluctuations may mask seasonal changes, especially in studies of free-living birds where confounding variables may be difficult to control.

Gonadotropin releasing hormone (GnRH) bioassays in which exogenous GnRH is administered, sometimes called GnRH challenges, are used to determine the physiological capacity of an individual to elevate T (Wingfield et al. 1979; Wingfield et al. 1991; Schoech et al. 1996; Jawor et al. 2006) termed the $R_{\text{potential}}$ of an individual (Goymann et al. 2007). Through the administration of a large dose of GnRH, researchers, in essence, flood the GnRH receptors of the anterior pituitary (Wingfield et al. 1991; Goymann and Wingfield 2004; Bentley et al. 2006). The subsequent release of LH into circulation effectively tests the ability of the gonads to elevate T ($R_{\text{potential}}$) and, since this approach requires only a single
capture, it is easy to use with free-living individuals (e.g. DeVries et al., 2012; Devries et al., 2011).

In this study I sought to investigate HPG axis activity in male and female birds during their spring migration. To do so I determined the $R_{\text{potential}}$ in Swainson’s Thrushes (Catharus ustulatus; hereafter thrushes) at different points along their migratory route as they traveled towards their breeding grounds. Although an earlier study did not find changes in the seasonal levels of T in this species during migration (Covino et al. 2015), I predicted that birds’ ability to elevate T (or $R_{\text{potential}}$) would increase during migration (Goymann et al. 2007). Specifically, I hypothesized that the $R_{\text{potential}}$, as determined by levels of T in response to GnRH injection, would increase throughout the migratory period as birds move closer to their breeding destination, where higher levels of T are required for the transition into breeding behaviors. Given that both sexes must be prepared for breeding activities and behaviors upon arrival on the breeding grounds, I also predicted that both males and females would have a higher $R_{\text{potential}}$ when sampled closer to their breeding grounds.

Materials and Methods

Study Species and Study Sites

My focal species was the Swainson’s Thrush, a long-distance Nearctic-Neotropical migrant that breeds throughout the boreal forests of North America and winters in southern Central America and eastern South America (Figure 10). As in Covino et al., (2015) I captured migrating thrushes throughout vernal migration at one southern site (from 6 April through 12 May) and one northern
site (from 10 May through 2 June) during 2012-2014. My study encompassed the entirety of thrush migration at both study locations. My southern site is relatively far (at least 1,400 km to closest point on breeding range) from the breeding grounds for this species and is located along the northern coast of the Gulf of Mexico in Cameron Parish, Louisiana (29° 45’ N 93° 30’ W). My northern site is relatively close (less than 200 km to closest breeding range) to the breeding grounds for this species and is located on an island off the New England coast in York County, Maine (42°58’ N, 70°36’ W; Figure 10). I also captured birds at a site along the Gulf of Mexico coast in Mobile County, Alabama (30° 15’ N 88° 05’ W), during fall migration from 20 September through 8 October, 2014, to enable sample comparisons outside of either spring migration or the breeding season. 

Capture and Sampling

Birds were captured using mist-nets which were operated daily, weather permitting, throughout each migratory season. Within 10 minutes (Mean ± SD; 4.6 ± 1.9 min) of a bird’s removal from a net, I obtained a blood sample (approximately 70 - 100 μl) via the brachial vein with a 26 gauge needle and collected blood into heparinized capillary tubes. After initial sampling, I administered a 100 μl injection into the pectoralis major of either 1 M phosphate-buffered saline (PBS; control birds; N = 38) or a GnRH solution made from 2.5 μg of chicken GnRH-I (American Peptide Co., #54-8-23; N = 182) dissolved in 100 μl of PBS (following Jawor et al., 2007, 2006). This dosage of GnRH has been used previously to stimulate T production in a similarly sized species, the Northern Cardinal (Cardinalis cardinalis; DeVries et al., 2012, 2011). Injected
birds were held in either a wooden holding box or a cloth bag until a post-injection blood sample was obtained. Based on the results from a small study (N = 22) to determine the timing of R_{potential} in which we sampled thrushes during spring migration at our southern study site at various time points post-injection (see Results), I held for 30 minutes between administering the injection and obtaining the post-injection sample. After completion of the GnRH bioassay, each bird was banded with a USGS aluminum leg band and measurements of wing length (unflattened wing chord; nearest 0.5 mm), and body mass (nearest 0.01 g) were taken. All blood samples were kept in a cooler on ice until they were centrifuged at 14,000 rpm for 10 minutes. The plasma portion was drawn off and stored at -20°C and the cellular portion was placed in approximately 500 μl of lysis buffer (50 mM TRIS, 10 mM EDTA, 1% SDS, 0.1 M NaCl) and stored at either 4°C or -20°C. As described in Covino et al., (2015) the cellular portion was used to determine sex genetically based on methods in Griffiths et al. (1998, 1996).

**Testosterone Assays**

I used an enzyme immunoassay to determine T levels (Enzo Life Sciences Inc., #901-065; antibody sensitivity = 5.67 pg/ml; (Jawor 2007; DeVries et al. 2011; Covino et al. 2015). Tritium-labeled T (approximately 2000 cpm; PerkinElmer) was added to 25-30 μl of each plasma sample (depending on available sample volume). After three extractions with diethyl ether, samples were re-suspended with 50 μl of ethanol and 300 μl of the assay buffer provided in the assay kit. Samples were run in duplicate on the assay plate using 100 μl of
the reconstituted sample each. Recoveries were determined with 100 μl from each reconstituted sample (mean recoveries = 85%). After correcting for incomplete recoveries and initial plasma volume, a logistic curve was used to determine T concentrations (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, California). Intra- and inter-assay variations were determined based on three standard samples of known T concentration placed in each assay plate (N = 19). Intra-assay variation ranged from 0.7% to 11.5% and inter-assay variation was 12.0%. Inter-assay variation may have been inflated due to the use of EIA kits from multiple kit lots.

**Statistical Analyses**

Based on the standards placed within each assay plate, T data were corrected for inter-assay variation before analyses and were cube-root transformed to compensate for their deviation from a normal distribution. For analyses of seasonal variation in the GnRH response, data were organized into four sampling periods: fall migration, south-early spring migration, south-late spring migration, and north spring migration. South represents birds sampled in Louisiana and north represents birds captured in Maine. Louisiana birds were categorized as ‘early’ or ‘late’ based on when they were captured relative to the median date of capture for each sex because the migratory period is relatively protracted there compared to Maine.
Figure 10. The breeding and wintering range of the Swainson’s Thrush (Catharus ustulatus).

Note: The breeding range is limited to the boreal forests of North America and the wintering range extends from Central American through western South America. The sampling locations in Louisiana and Maine are indicated. This map was generated using data from BirdLife International (BirdLife-International and NatureServe 2014).

To estimate both interactive and main effects, I used linear mixed effects models which allow for modeling of random effects (Bates 2010; Bates et al. 2014). To determine the significance of model interaction terms, I compared models with interactions to those without them using likelihood ratio tests. I also used likelihood ratio tests to determine the significance of fixed effects by comparing full models to similar models without the variable in question. I used
repeated measures models structured with individual as the random effect in three sets of analyses. (1) A time-series analysis for post-bleed time with T as the response variable and with sex and sample (0, 15, 30, 45, 60 min) as fixed effects. (2) An analysis of control (PBS injected) birds with T as the response variable and with sampling period (spring only) and sample (pre- or post-injection) as fixed effects. In this analysis, males and females were analyzed separately. (3) An analysis of GnRH-injected birds with T as the response variable and with sampling period, sample (pre- or post-injection), and sex as fixed effects. A fourth analysis investigated temporal variation across spring migration. In that analysis, I calculated $R_{\text{potential}}$ to the GnRH bioassay as the post-injection T level less the pre-injection T level for each individual (Goymann et al. 2007). I analyzed $R_{\text{potential}}$ for males and females separately using generalized linear models with date (day of year) as the predictor variable with data pooled across both sites. As necessary, I analyzed pairwise effects using least squares means tests with a Tukey correction for multiple comparisons (R package lsmeans).

Results

Controls and Time Series Trials

Post-GnRH-injection T levels depended upon the time when the post-injection sample was taken ($\chi^2 = 19.48$, df = 3, $P < 0.001$). In both males and females, samples taken at 15, 45, or 60 minutes post-injection were no different from pre-injection samples ($P > 0.80$ for each pairwise comparison; Figure 11). Samples taken at 30 minutes post-injection were significantly higher than initial T
levels in both sexes (P < 0.05 for each). There was no difference between T levels before and after PBS injection in either control males (N = 24; χ² = 3.2, df = 1, P = 0.08) or control females (N = 14; χ² = 0.03, df = 1, P = 0.87) regardless of sampling period/site (Figure 12).

Figure 11. Time trials for GnRH injections in Swainson’s Thrushes.

Note: Testosterone levels in Swainson’s Thrushes before (Time = 0) and at various time points after an injection of GnRH administered during spring migration in Louisiana. Error bars represent standard error of the mean. Sample sizes within each sex and time point are provided below the error bars.

Seasonal Variation in GnRH Response

Third-order interactions were evident when data from males and females were analyzed together and sex was included as a fixed effect (χ² = 41.00, df = 10, P < 0.0001), so data for males and females were analyzed separately for
subsequent analyses of T and sampling period. In males, there was a significant interaction between sample (pre- or post-injection) and sampling period on T ($\chi^2 = 22.23$, df = 3, $P < 0.0001$). Pairwise analyses revealed a number of differences (Figure 13). There was no pattern of increasing pre-injection (circulating) T levels across migratory stage (Figure 13). Post-injection T was lower in fall relative to all spring samples and was highest at the northern sampling location overall ($P < 0.01$ for each comparison). Additionally, post-injection T levels at the northern sampling location were higher than pre-injection (circulating) levels at all migratory stages ($P < 0.001$ for each comparison).

The effect of the injection on T was consistent across sampling period in females ($\chi^2 = 3.03$, df = 3, $P = 0.39$; Figure 14). There was a significant effect of sample (pre- or post-injection) where post-injections samples were overall higher than pre-injection samples ($\chi^2 = 66.30$, df = 1, $P < 0.0001$). Migratory stage also had an overall fixed effect on T ($\chi^2 = 15.63$, df = 3, $P < 0.01$). As determined by pairwise evaluation of effects, pre-injection T levels were consistent across the three spring sampling periods (Figure 14; $P > 0.50$ for each comparison), but all values were higher than pre-injection levels during the fall sampling period ($P < 0.05$ for each comparison). Post-injection levels were higher than pre-injection levels during the spring sampling periods but not during the fall. While higher than the post-injection levels during fall ($P < 0.05$ for each comparison), post-injection levels across all three spring sampling periods were similar ($P > 0.90$ for each comparison).
Figure 12. Testosterone levels in male and female migrating Swainson’s Thrushes before and 30 minutes after an injection of phosphate-buffered saline.

Note: Pre-injection levels are represented by the open circles and post-injection levels by filled circles. Sampling periods correspond to migration season (fall or spring), the location of sampling (see Figure 10), and/or the time period of passage. Sample sizes within each sampling period are provided. There was no difference in testosterone levels between pre-injection and post-injection samples.

Temporal Variation

In males, $R_{potential}$ (response to GnRH injection) increased with date such that many individuals had low or no response early in the spring season while individuals sampled later had a fairly strong response ($t = 3.81$, df = 81, $P < 0.001$; Figure 15). $R_{potential}$ did not change with date in females ($t = 0.78$, df = 89, $P = 0.44$; Figure 15).
Figure 13. Testosterone levels in male migrating Swainson’s Thrushes before and 30 minutes after an injection of GnRH.

Note: Pre-injection levels are represented by open circles and post-injection levels by filled circles. Back transformed means and standard errors are presented. Sampling periods corresponds to migration season (fall or spring), the location of sampling (see Figure 10), and/or the time period of passage. Sample sizes within each sampling period are provided. Groups that are significantly different are indicated with different capital letters as determined by least squares means pairwise comparisons.
Figure 14. Testosterone levels in female migrating Swainson’s Thrushes before and 30 minutes after an injection of GnRH.

Note: Pre-injection levels are represented by open circles and post-injection levels by filled circles. Back transformed means and standard errors are presented. Sampling periods corresponds to migration season (fall or spring), the location of sampling (see Figure 10), and/or the time period of passage. Sample sizes within each sampling period are provided. Groups that are significantly different are indicated with different capital letters as determined by least squares means pairwise comparisons.
Figure 15. Testosterone response to gonadotropin-releasing hormone bioassays relative to date in migrating Swainson’s Thrushes.

Note: Data for (A) male and (B) female Swainson’s Thrushes sampled during spring migration for both study locations combined are presented. Response was calculated as the levels of testosterone 30 minutes after an injection with gonadotropin-releasing hormone minus initial testosterone levels. Solid lines indicate the best fit relationship and the shaded areas are the 95% confidence intervals.

Discussion

I examined HPG axis activity in male and female thrushes during spring passage to investigate the degree of overlap the developmental phase of breeding and mature expression of spring migration in songbirds. Male thrushes responded to a GnRH injection with increases in T levels, and the magnitude of this $R_{potential}$ increased both over time and with geographic proximity to the breeding grounds clearly indicating that the central mechanisms are preparing for
breeding during spring migration in males. The GnRH bioassays elicited significant elevations in T levels in female thrushes but their $R_{\text{potential}}$ remained constant throughout spring migration. The results reveal overlap between preparation for breeding and spring migration in male songbirds, while the pattern in females may indicate a more conservative strategy and raises important questions about the transition from migration to breeding in female migratory songbirds.

In my examination of HPG axis activity in Black-and-white Warblers (*Mniotilta varia*) at a single migratory site where individuals were heading to a range of breeding destinations (Chapter III), I found a similar sex-dependent pattern: Males closer to their breeding destination showed greater $R_{\text{potential}}$ compared to those that were relatively further away but $R_{\text{potential}}$ was unrelated to breeding ground proximity in females. Bluhm et al. (1991) detected evidence of increasing HPG axis activity in male Garden Warblers (*Sylvia borin*) as they progressed from photorefractory to photosensitivity based on increasing hypothalamic GnRH and pituitary LH content and plasma LH on constant photoperiodic conditions. While Wingfield and Farner, (1978b) found similar increases in LH in migrating male White-crowned Sparrows (*Zonotrichia leucophrys gambelii*), Bauchinger et al. (2007) did not detect any changes in the LH levels of migrating Garden Warblers. Moreover, testicular recrudescence has also been shown to occur throughout spring migration in both free-living (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Bauchinger et al. 2005; Bauchinger et al. 2007) and captive migrants under long day photoperiods.
(Bluhm et al. 1991; Wingfield et al. 1996; Wingfield et al. 1997). Collectively studies focused on non-stimulated circulating T during migration have yielded conflicting results regarding whether T levels increase in male songbirds during spring migration (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Bluhm et al. 1991; Bauchinger et al. 2007; Covino et al. 2015). However, investigations of HPG axis activity via measures of hypothalamic GnRH, gonadotropins, or using GnRH bioassays, and gonad recrudescence provide strong evidence for the overlap between spring migration and breeding development. Seemingly by increasing HPG axis activity without substantial increases in general circulating T levels, a balance is created between the necessity for breeding preparation to occur during spring migration and the potential antagonistic effects of high levels of T on migration itself.

Evidence suggests that some degree of breeding preparation occurs either before or during spring migration in female songbirds. That said, research has failed to produce consistent results across all hormones investigated, including T, LH, FSH, and estradiol, or across every species studied (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Wingfield et al. 1996; Wingfield et al. 1997; Covino et al. 2015). For example, in Wingfield and Farner’s (1978a) study of female White-crowned Sparrows (Z. l. pugetensis), the authors detected increases in both T and estradiol but not LH. Additionally while Covino et al. (2015) showed that T levels increased during migration in female Northern Waterthrushes (Parkesia noveboracensis), this pattern was not found in the two other songbird species including the Swainson’s Thrush. In the current study,
even though the degree to which female Swainson’s Thrushes could elevate T did not vary throughout spring migration, their ability to elevate T significantly during spring migration contrasted with responses during other non-breeding time periods (i.e. fall migration). Some studies have demonstrated that the initial stages of follicular development occur during the migratory period with the caveat that estradiol stimulation for final maturation of the ovary, yolk production, and yolk deposition in the follicles does not occur until females reach the breeding grounds (Wingfield and Farner 1978a; Wingfield and Farner 1978b). My study and those centered on ovarian development indicate that while females show gonadal development prior to the breeding season, the full progression of increasing HPG axis activity and ovarian development may be delayed, in part, until females arrive on the breeding grounds and can assess local environmental and social conditions prior to breeding (discussed below).

The initial predictive cue for HPG axis activation and the development of migratory traits in both male and female songbirds is photoperiod (Rowan 1925; Ball and Ketterson 2008; Helm et al. 2009; Ramenofsky 2011). In addition, supplementary factors, temperature and behavioral interactions, provide fine tuning of the HPG axis for complete maturation of the female reproductive cycle (Ball and Ketterson 2008). In my study, while $R_{\text{potential}}$ in female thrushes did not vary during migration, $R_{\text{potential}}$ in males increased as the migratory season progressed. Thus males and females may be employing different strategies regarding the overlap between spring migration and the development of breeding. In males, development occurs at a steady pace throughout the
migratory period, ensuring they arrive on the breeding grounds prepared to fully express breeding traits. Females, however, likely employ a more conservative strategy by requiring supplementary and local from the breeding grounds thus delaying the final stages of breeding preparation until arrival. The supplementary cues that regulate HPG axis action in females may include behavioral interactions with courting males as well as environmental cues such as temperature and food availability (Ball and Ketterson 2008). Thus the final stages of HPG axis activation and ovarian development may be delayed until females reach the breeding grounds where these supplementary cues would be available to them.

Both male and female thrushes had higher $R_{\text{potential}}$ during spring migration than fall migration. While sample sizes for fall birds were low, these results support the idea that T and HPG axis activity are increasing during spring migration as a function of photoinduction and breeding preparation rather than due to their necessity for migration itself. Similarly, in some migratory species, migratory behaviors have been expressed prior to gonadal recrudescence indicating an asynchrony between the development of migratory and breeding characteristics (Hahn et al. 1995). In fact, the photoperiodic cues for development of spring migration and breeding may be separate (Wang et al. 2013) thus development of one stage (e.g. migration) would not be required for the development of the subsequent stage (e.g. breeding). In this study, T levels did not elevate in response to the GnRH bioassay in either male or female
thrushes tested during fall migration indicating that this ability is not a requirement for migration.

Conclusion

My findings indicate that the schedule for HPG axis activity is different for migrating male and female thrushes. Similar results were found in a different long-distance migratory songbird, the Black-and-white Warbler (Chapter III), suggesting that this pattern may have wide applicability. In both studies, $R_{\text{potential}}$ increased during the migratory period in male migrants. While the GnRH bioassays generally elicited a response in female migrants, $R_{\text{potential}}$ remained steady throughout the migratory period. One potential explanation for this phenological difference between males and females is the sex-specific differences in the breeding development strategies. Due to the need for supplementary cues from the breeding grounds, the final stages of HPG axis activation may be delayed until arrival in female songbirds.
CHAPTER V – MIGRATORY STOPOVER BIOLOGY NOT RELATED TO CIRCULATING TESTOSTERONE LEVELS

Abstract

For migratory birds, a successful journey is dependent upon performance during migratory stopover as well as during migratory flight. During spring, migrating songbirds begin the development of breeding physiology, which may include increases in testosterone levels, as they approach the breeding grounds. Testosterone is an influential hormone mediating effects throughout the breeding season but its role during spring migration is understudied. I investigated the relationship between testosterone and stopover likelihood, stopover length, foraging behaviors, fuel deposition rate, and intensity of competition from other migrants in Black-and-white Warblers (Mniotilta varia) at a spring stopover site. Testosterone was unrelated to all measures of stopover biology. I also investigated variation in testosterone during the stopover period and found that testosterone was lower in birds after they had been on the site for 2-14 days. In order to fully investigate the potential relationship between testosterone and stopover biology, future studies should experimentally manipulate hormone levels in free-living migrants.

Introduction

Migration is a widespread phenomenon which has evolved as an adaptation to exploit seasonal and geographic variation in abundance of resources (Dingle 1996; Dingle 2006). The majority of migratory birds must utilize short stopovers to rest and refuel in between bouts of migratory flight and it is
during these periods of stopover where the majority of time and energy is spent (Hedenstrom and Alerstam 1997). Long-distance migrants spend approximately one quarter to one half of the annual cycle traveling between breeding and wintering areas (Keast 1980; Lindström 2005). These seasonal migratory periods are energetically challenging and must be balanced with demands of other phases of the annual cycle (Newton 2011). Performance during stopover may not only determine the success of the overall journey but also contribute to carry-over effects that may influence other stages of the annual cycle (Moore et al. 2005; Marra et al. 2015).

In order to be successful on stopover, migrants must acquire the resources necessary to recover from and refuel for migratory flights. Testosterone (T) is usually characterized as a breeding hormone (Balthazart 1983; Wingfield et al. 2001). In addition to mediating physiology and behavior related to breeding T also influences initiation of hyperphagia and fueling in both male and female songbirds (e.g. Stetson and Erickson 1972; Schwabl and Farner 1989a; reviewed in Deviche 1995). Lynn et al. (2000) found that breeding male Dark-eyed Juncos (Junco hyemalis) with experimentally elevated T moved at faster rates and spent more time foraging than controls, while daily energy expenditure remained the same. Studies have suggested that T is linked not only to pre-migratory fattening and mass increases, but also to hyperphagia which promotes increases in muscle and fat needed for migration (reviewed in Deviche 1995). Two recent studies demonstrate that captive male birds with experimentally elevated T exhibit migratory hyperphagia, mass gains, and
migratory restlessness earlier than control individuals (Tonra et al. 2011a; Owen et al. 2014). Studies have also shown that removal of the gonads, and the resulting reduction in T production, prevents both male and female birds from fattening (Stetson and Erickson 1972; Schwabl and Farner 1989a; Schwabl and Farner 1989b). Exogenous replacement of T reverses this effect, allowing birds to fatten and increase in mass. However, while Wikelski et al. (1999) found that elevated T led to an increase in activity and food intake by captive White-crowned Sparrows (Zonotrichia leucophrys gambelii), high T was also linked to decreases in body mass. Additionally, Chandler et al. (1994) did not see a difference in the time spent foraging between male Dark-eyed Juncos (Junco hymealis) with elevated T and controls during the breeding season. While the relationship between T and foraging/feeding behaviors in songbirds may not be entirely clear, we know even less about how T may impact the biology of birds during migratory stopover. Measures of food intake are common in studies of captive migrants (e.g. McWilliams and Karasov 1998; Heise and Moore 2003; Landys et al. 2004; Holberton et al. 2008) including those evaluating T’s role in migration (e.g. Deviche 1992; Tonra et al. 2011), yet no study to date has evaluated potential correlations between T and foraging behaviors or fuel deposition rates in migrants on stopover.

When migrants with heightened energy demands are locally concentrated in unfamiliar areas, competition for available food resources could reduce the rate at which some individuals meet nutritional requirements (Carpenter et al. 1983; Hansson and Pettersson 1989; Moore and Yong 1991). Testosterone is
well known for its role in mediating inter- and intra-specific aggression and territorial defense in songbirds during the breeding season (cf. Balthazart 1983; Wingfield et al. 2001). For example, Chandler et al. (1994) found that male Dark-eyed Juncos with experimentally elevated T had larger territories, spent more time singing, and less time at the nest (attending to eggs/nestlings) than controls. Additionally, patterns of circulating T are tightly associated with territoriality in migratory White-crowned Sparrows, but Song Sparrows (*Melospiza melodia*), a non-migratory species, sometimes display territoriality even when T levels are low (Wingfield and Hahn 1994). Since T may influence behaviors such as aggression during breeding as well as at other times of the year, one may expect to see a correlation between T and aggressive behaviors when migrants are competing for limited resources during stopover (but see Schwabl et al. 1988a; Ramenofsky et al. 1992).

Physiological changes associated with breeding preparation have long been linked to the expression of migratory traits (Rowan 1925; Rowan 1929; Stetson and Erickson 1972; Schwabl and Farner 1989a; Schwabl and Farner 1989b). Several studies have demonstrated that long-distance migratory songbirds begin preparation for breeding during spring migration and that T levels may also increase throughout the migratory period (Wingfield and Farner 1978a; Wingfield and Farner 1978b; Covino et al. 2015; Chapter III; but see Wang et al. 2013). Transitory changes in T levels may result from hormone response to a wide variety of environmental or social factors (Wingfield et al. 1990; Goymann et al. 2007). For example, T levels decreased in migrants that
experienced food deprivation to imitate fasting during long flights (Bauchinger et al. 2009). Additionally, some studies have indicated that corticosterone may suppress T (Deviche et al. 2001; Swett and Breuner 2008) and research has indicated that corticosterone may increase during migratory flight in both shorebirds (Landys-Ciannelli et al. 2002; Reneerkens et al. 2002) and songbirds (Ramenofsky et al. 2008; Falsone et al. 2009). Thus we might expect T levels to become suppressed in response to long migratory flight and therefore be lower upon arrival at a stopover site and slowly recover throughout the stopover period.

This study focuses on the relationship between T and the biology of migratory birds during stopover. Specifically I determined whether there is a relationship between T and (1) likelihood and duration of stopover, (2) foraging behaviors (movement rate and maneuver rate), (3) intensity of competition from other migrants, and (4) fuel deposition rate during stopover. Additionally, I investigated whether T levels changed throughout the stopover period. I predicted that foraging rates, intensity of competition, and fuel deposition rate would be positively correlated with circulating T levels. Since these measures make for a more successful migrant and since previous research demonstrates that T may advance the schedule for migration, I predicted that birds with higher T levels would be less likely to remain on stopover, and if they did, would have shorter stopover durations. I also predicted that T levels would be higher in recaptured individuals that have been on stopover for a few days compared to what they were upon initial capture.
Methods

Study Species and Study Site

The study site was located along the northern Gulf of Mexico coast in Cameron Parish, Louisiana (29° 45’ N 93° 30’ W). This coastal woodland is dominated by Hackberry (*Celtus laevigata*), Live Oak (*Quercus virginiana*), and Honey Locust (*Gleditsia triacanthos*) extending for approximately 20 miles along the coast and surrounded by marsh. Research efforts were focused within a two hectare section of forest where migrants were captured after completing a flight across the Gulf of Mexico. This trans-Gulf flight takes approximately 16-20 hours to complete (Moore et al. 1990) and peak arrival occurs from 10:00-15:00 at this study site but may be delayed until 17:00-18:00 depending on wind conditions (FRM unpublished data). Additionally, migrants are generally captured soon after they arrive on site (KMC personal observation).

The focal species, the Black-and-white Warbler (*Mniotilta varia*; BAWW), is a Nearctic-Neotropical migrant with a wintering range extending through Central America, the northern coast of South America, as well as the Antilles, Florida, and east coastal Texas (Kricher 2014). The BAWW breeding range extends from the southeastern United States throughout most of eastern North America and the boreal forest of Canada (Figure 16). BAWW is an insectivore with a diet comprised primarily of lepidopteran larvae as well as other terrestrial arthropods (Bent 1953). This species has a fairly unique “tree-creeping” foraging pattern for a parulid warbler (Kricher 2014) making it relatively easy to visually identify in the field and thus ideal for behavioral studies.
Figure 16. Breeding and wintering range for the Black-and-white Warbler (*Mniotilta varia*).

Note: The breeding range (green) and wintering range (blue) are specified. This map was generated using data from BirdLife International (BirdLife-International and NatureServe 2014).

**Capture and Sampling**

Migrants were captured using up to 29 mist-nets, weather permitting, morning through evening throughout spring migration, 18 March through 12 May, 2012-2014. After capture BAWW were transported to a central processing location where a small blood sample (~60 μl) was obtained via the brachial vein within 10 minutes of removal from a net (Mean ± S.D.; 4.9 min ± 2.1). Samples were collected into heparinized capillary tubes and then stored on ice in a cooler
for the remainder of the field day. Samples were later centrifuged and the plasma portion was separated and stored at -20°C. BAWW were banded with a USGS leg band and given a unique combination of three color bands allowing for future visual identification. By examining feather wear and molt limits, age was determined as either second year (SY), indicating a bird undertaking its first spring migration, or after-second year (ASY; Pyle 1997). Sex was determined using plumage characteristics (Pyle 1997); wing length and body mass were also measured and recorded.

The average mass of BAWW in this study was 9.5 g (± 0.92 SD) and thus caution regarding blood sampling volume was necessary (see Owen 2011). Some individuals had a larger volume taken upon first capture because of their inclusion in a previous study (Chapter III) and thus were not included in aspects of this study that required recapture and a subsequent blood sample. A second blood sample was obtained from an individual upon its recapture only if (1) blood sample volume upon initial capture did not exceed 0.7% of the bird’s measured body mass and (2) the recapture occurred at least 48 hours after the initial blood sample was obtained. Birds from which I obtained second blood samples did not show visible signs of stress nor was obtaining the sample difficult relative to obtaining an initial blood sample (KMC personal observation). To ensure that obtaining a second sample upon recapture did not influence my stopover measures, individuals from which a recapture sample was obtained were not included in analyses of stopover likelihood, stopover duration, or fuel deposition
rate. Further, T levels from initial samples, those taken upon first capture, were used in analyses of foraging behaviors and competition.

*Re-sightings and Behavioral Observations*

Re-sighting of color banded individuals was attempted each day throughout the 2013 and 2014 field seasons for a minimum of 2 hours regardless of weather conditions or other field/banding activities. Typically 6-11 hours were spent searching for marked BAWW each day. When an individual was re-sighted, its color combination was noted and later used to determine the individual’s identity. A trained technician then attempted to record the individual’s foraging behaviors using the methods in Remsen and Robinson (1990). The bird was observed through either 10 x 50 or 8 x 42 binoculars while the type and distance of each movement or foraging maneuver, the substrate, interactions with other individuals, and food type were recorded digitally. Observations were at least 20 seconds but no more than 2 minutes in length (Hejl and Verner 1990; Kleintjes and Dahlsten 1995) and were transcribed for calculation of both movement rate (meters per minute) and foraging maneuver rate (maneuvers per minute). Time spent performing non-foraging activities (preening, resting, etc.) was not included in these calculations.

*Testosterone Determination*

Plasma T levels were determined using enzyme immunoassays (Enzo Life Sciences Inc. #901-065; antibody sensitivity = 5.67 pg/ml) as has been described in (Jawor et al. 2007; Covino et al. 2015). Tritium labeled T (2000 cpm) was added to 20-40 μl of plasma to allow for calculation of recoveries after three
extractions with diethyl ether. The EIA kit assay buffer and ethanol were used to re-suspend the extracts and then samples were run in duplicate in the EIA. A logistic curve was used to determine T concentrations (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, California) which were then corrected for initial plasma volume and recovery percentage. Assay variation was determined using three standard samples of known T on each of the 16 assay plates used in this study. Intra-assay variation ranged from 0.65% to 10.5% and inter-assay variation was 12.4%.

*Feather Hydrogen Determination*

Prior work has indicated that the BAWW that pass through this study site are heading to a wide range of breeding destinations extending from the southeast through the northern boreal forests of North America (Paxton and Moore 2015; Chapter III; Figure 1). So I incorporated an evaluation of breeding destination in this study by determining the hydrogen isotope ratio in each individual’s feathers. BAWW undertake their prebasic molt on their breeding grounds during which either all feathers (adults) or only body feathers (young of the year) are replaced (Pyle 1997). Since young birds grow their other feathers, including their flight feathers, in the nest, the flight feathers for all individuals of this species represent the hydrogen isotope signature of an individual’s breeding location. The hydrogen isoscape primarily follows a north-south gradient in eastern North America, thus isotope values from feathers can be employed to represent breeding destination of sampled migrants throughout the annual cycle (Hobson and Wassenaar 1999; Kelly et al. 2002).
Two tail feathers (the right and left fifth rectrices) were pulled from each bird at the time of capture and were stored until processing. Feather hydrogen values were determined as detailed in Chapter III. Briefly, feathers were first cleaned with an immersion in 2:1 chloroform:methanol for 30 s (Paritte and Kelly 2009) and were then allowed to air dry for 24 h under a fume hood. They were cleaned with a 1:30 solution of detergent and deionized water, rinsed three times in deionized water, and allowed to dry again for 24 h under a fume hood. The samples were weighed and 350 ug ± 10 of each was loaded into 3.5 mm × 5 mm silver capsules. Along with duplicate samples, three standards were also analyzed as follows: (1) finely powdered feathers from a brown-headed cowbird (Kelly et al. 2009), (2) USGS-40, and (3) USGS-41, both purchased from the National Institute of Standards and Technologies. Samples were sent to the Colorado Plateau Stable Isotope Facility for mass spectroscopy analyses and were corrected for instrumental drift within runs. Feather hydrogen data are presented as ($\delta^2$H) which denotes the ratio of heavy ($^2$H) to light ($^1$H) isotopes in each sample. Higher values thus represent less depleted samples with relatively more of the heavier isotope and indicate a bird’s breeding destination is at the southerly part of the range.

**Data Preparation**

Prior to data analysis, several metrics were calculated and data corrections were made. Testosterone levels were corrected for inter-assay variation using the mean of the three T standards of each assay. Further, T data did not meet normality assumptions but a cube-root transformation corrected this
and was used for all analyses. Stopover likelihood was represented as a binomial based on whether each individual was seen (re-sighted or re-captured) on the study site on any day subsequent to the day it was initially captured. Minimum stopover length was calculated as the number of days between a bird’s first capture and when it was last encountered on the site either from a recapture or re-sighting (Cherry 1982; Moore and Kerlinger 1987). Energetic condition was calculated as the difference between each individual’s size-specific fat-free mass which was determined using a large dataset \( (N = 1,145) \) of BAWW (Owen and Moore 2006; Covino et al. 2015). Negative values for energetic condition indicate a bird whose mass is less than what is a typical fat-free mass for a bird of that size (wing length) and is thus in poor condition. Positive values indicate a bird whose mass is greater than its fat-free mass and is in good condition.

Fuel deposition rate was calculated as the change in mass for an individual per day using methods similar to Cherry (1982). To ensure these data were not biased by the time of day birds were captured, first I calculated hourly fuel deposition rate for all BAWW that had been re-captured at the study site in previous years \( (1994-2012) \) on the same day as their initial capture \( (N = 34) \). This hourly fuel deposition rate was calculated as the final mass less the initial mass per hour between captures. Then the mean hourly fuel deposition rate was used to adjust the mass of each bird captured on any day subsequent to its initial capture to what it would be at 1900 since this time is shortly prior to sunset at this site and no birds were captured after this time. This correction was done by adding the product of the hourly fuel deposition rate and the difference between a
bird’s capture time and 1900. Finally, the adjusted mass values were used to calculate the corrected daily fuel deposition rate by taking the corrected mass from the last day of capture less the corrected mass from the first day of capture and dividing the number of days in between captures. Competition intensity was represented by the total number of all other migratory songbirds captured at the study site on the same day as each BAWW individual which ranged from 8 to 445.

Data Analyses

I used linear mixed models to investigate the relationship between T and stopover length, stopover likelihood, competition intensity, foraging movement rate, and foraging maneuver rate. I used a similar approach to investigate changes in testosterone during the stopover period but this analysis incorporated a repeated-measures design. For each analysis, I first elevated the potential for interactive effects between predictor variables by testing for significant differences between interactive models and equivalent additive models with likelihood ratio tests using the chi-squared distribution. I then tested for the significance of each predictor variable with likelihood ratio tests by comparing the full model to the model sans the variable being evaluated (see Bates 2010; Bates et al. 2014).

To analyze changes in T during stopover, I included sample (initial or recapture) and sex as predictor variables, T as the response variable, and individual and days between samplings as random variables. In my analyses of stopover length, stopover likelihood, foraging movement rate, and foraging
maneuver rate, date was treated as a random variable, and testosterone was evaluated as a predictor variable. I additionally evaluated sex, feather hydrogen, age, and energetic condition as potential covariates (Table 1). I fit models using the binomial distribution when analyzing stopover likelihood and used the Gaussian distribution for all other analyses.

To investigate the effects of competition intensity on testosterone, I evaluated the total number of migrants captured that day as a predictor variable along with feather hydrogen, and age as covariates. Based on the results of a previous study of BAWW indicating that date and time of day may be related to testosterone levels and/or feather hydrogen in this species (Chapter III), I included both date and time of day as random variables in this analysis. Likewise, since males and females show different patterns of varying testosterone levels (Chapter III), I analyzed the sexes separately in this investigation of competition intensity. Lastly, I used generalized linear models to investigate the potential effects of testosterone on fuel deposition rate and included both sex and feather hydrogen as covariates. All analyses were performed using Program R (R Core Team 2015) and I used package lme4 for the linear mixed models.

Results

Testosterone levels for BAWW in this study ranged from 0.006 ng/ml to over 5.0 ng/ml (0.006-5.03 ng/ml for males and 0.02-3.28 for females; Figure 17). Overall males had higher T than females ($\chi^2 = 4.19, \text{df} = 1, P = 0.04$); the median
T level for males was 0.98 ng/ml and females was 0.82 ng/ml (initial capture T values).

![Graph showing testosterone levels in male and female Black-and-white Warblers during spring migration.](image)

**Figure 17.** Testosterone levels in male and female Black-and-white Warblers during spring migration.

**Changes in Testosterone during Stopover**

I was able to obtain a blood sample from a total of 17 individuals (14 females and 3 males) that were recaptured at least 48 hours subsequent to their initial single sample. T levels were significantly lower in individuals sampled 2-14 days after their initial capture relative to those same individuals at first capture ($\chi^2 = 5.94$, df = 1, $P = 0.015$; Figure 18). These analyses controlled for the variable number of days between initial capture and recapture and a follow-up investigation did not find a relationship between the change in T levels and the number of days between samples ($t = 0.05$, df = 15, $P = 0.96$). While there was no interaction between sample (initial or recapture) and sex on T ($\chi^2 = 0.36$, df =...
males had overall higher T levels than females ($\chi^2 = 4.30$, df = 1, $P = 0.038$).

*Stopover Likelihood and Minimum Stopover Length*

Likelihood of stopover was not related to T nor was it related to any of the potential covariates investigated (Table 6). Additionally, overall model fit was poor ($< 30\%$ variance explained) indicating that the variables used in this study are not adequate predictors of stopover likelihood. Minimum stopover length and T were also unrelated (Table 6); however, I did find that the average minimum stopover length was over two days longer for females than males (5.0 days versus 2.9 days) and median stopover length was one day longer for females (3 days versus 2 days; Table 6; Figure 19). Additionally, birds in poor energetic condition had longer stopovers than those in better condition (Table 6; Figure 20).

*Testosterone and Competition*

Since patterns of T variation may be different between male and female BAWW (Chapter III), and given that T was the response variable in this set of analyses, I investigated the potential relationship between T and intensity of competition separately for each sex. Intensity of competition, as represented by the number of other migrants at the stopover site on the day of sampling, was unrelated to T levels in either male or female BAWW (Table 6).
Table 6

Linear mixed model analyses of five measures of stopover biology and behavior in Black-and-white warblers during spring migration

<table>
<thead>
<tr>
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<th>Fixed Effect</th>
<th>Model Statistics</th>
<th>Comparison to Full Model</th>
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Table 7 (continued).

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Note: Significance of predictor variables were determined via likelihood ratio test comparing the full model to the models sans the variable of interest. P-values were produced using the Chi-squared distribution and an asterisk signifies the significance of a variable. Samples sizes and model statistics for each analysis are provided.
Figure 18. Testosterone levels at initial capture and upon recapture of Black-and-white Warblers during spring migration.

Note: Birds were recaptured 2-14 days after their initial capture. Panel A depicts the change in testosterone levels for all 17 individuals with each individual’s initial and recapture values connected by a line. Females are denoted by the dashed pink lines and males by the solid blue lines. Panel B is a box and whisker plot depicting the median (middle solid line), the first and third quartiles (the box) and the range (whiskers) for the pooled initial and recapture testosterone levels.

Foraging Behaviors

The foraging behaviors of 50 individuals were observed throughout this study, however, I only analyzed foraging data from individuals whose observation was obtained within 24 hours of sampling (N = 38) because T levels may change over time (see results for ‘Changes in testosterone during stopover’ above). Circulating T was unrelated to foraging maneuver or movement rates (Table 6). Additionally foraging behaviors were not influenced by age or sex (Table 6). However, individuals with less depleted feather hydrogen values, indicating a more southerly breeding destination, moved faster and had higher foraging
maneuver rates compared to birds heading to more northerly breeding grounds (Table 6).

Figure 19. Minimum stopover length in male and female Black-and-white Warblers during spring migration.

Note: Minimum stopover length was calculated as the difference between a bird’s first capture and its last capture or sighting at a spring stopover site. Each quartile is represented by the box and whisker plot with the median as the central line. Extreme values are denoted by the solid points. Median stopover length for females was 1 day longer than for males.

Fuel Deposition Rate

Fuel deposition rate was calculated for 52 individuals that were captured at least once after initial capture and for which I also had both T and feather hydrogen data. Fuel deposition rate was not related to T levels ($t = 0.30$, df = 51, $P = 0.77$), and there was no difference between males and females in their fuel
deposition rate (t = 0.08, df = 51, P = 0.93). Additionally, fuel deposition rate was unrelated to feather hydrogen (t = 1.38, df = 51, P = 0.18).

Figure 20. Minimum stopover length in relation to energetic condition in Black-and-white Warblers during spring migration.

Note: Birds in poor condition had longer stopovers than birds in better condition.

Discussion

Testosterone levels in BAWW during stopover approached levels typical for breeding songbirds. Although there are no reported studies on T in BAWW, levels for songbirds during the breeding season generally range from 1-5 ng/ml in males and 0-2 ng/ml in females (reviewed in Ketterson et al. 2005). Testosterone levels in BAWW correspond to the lower end of the range for breeding songbirds, but within expected values for non-breeding songbirds (e.g. Tonra et al. 2013; see Ketterson et al. 2005). Additionally, T levels in male BAWW were similar to,
although slightly higher than, those measured in male American Redstarts
\((Setophaga ruticilla)\) upon arrival on the breeding grounds (Tonra et al. 2011b).

We know that T mediates aggression in both breeding and non-breeding
birds (Balthazart 1983; Wingfield et al. 2001) and may increase in response to
competitive or territorial threat during the breeding season (Wingfield et al. 1990;
Wingfield et al. 2000). In this study, however, I found T to be unrelated to the
density of migrants and presumed intensity of competition experienced by
BAWW during spring stopover. This result was similar to my work on the
Swainson’s Thrush \((Catharus ustulatus)\), Northern Waterthrush \((Parkesia
noveboracensis)\), and Magnolia Warbler \((Setophaga magnolia; Covino et al.
2015; Chapter II). It is important to keep in mind that circulating plasma T is not
always associated with aggression in non-breeding birds: several studies of
wintering male (e.g. Schwabl 1992; Wingfield and Hahn 1994; Soma et al. 2002;
Geslin et al. 2004) and female (e.g. Kriner and Schwabl 1991; Schwabl 1992;
Gwinner et al. 1994) songbirds have determined that territorial behaviors can be
exhibited independent of circulating plasma T (but Geslin et al. 2004). It is
possible that locally synthesized steroids, like estradiol in the brain, may be
mediating non-breeding aggression while circulating T levels are kept low
(Wingfield et al. 1997; Soma 2006). Clearly additional studies of aggression
during all non-breeding periods (i.e. wintering and migration) that include both
males and females are necessary to fully understand how these behaviors are
organized throughout the annual cycle.
The foraging behaviors and fuel deposition rates of BAWW did not vary with changing levels of T. Others have reported that hyperphagia and fat, and/or mass changes are linked to T in studies of captive songbird migrants where T was manipulated either via implants or removal of the gonads (Stetson and Erickson 1972; Schwabl and Farner 1989a; Schwabl and Farner 1989b). Moreover, experimentally elevated levels of T induced migratory disposition in captive Dark-eyed Juncos (*Junco hyemalis*; Tonra et al. 2011a) and Grey Catbirds (*Dumetella carolinensis*; Owen et al. 2014). However in Tonra et al. (2011a), while experimental birds exhibited an earlier onset of migratory disposition (as determined by measures of migratory restlessness, mass, and food intake) the peak levels of these metrics did not differ between experimental (elevated T) and control birds. Further, in their study, androgen (testosterone) levels of experimental birds were maintained at levels that greatly exceeded the un-manipulated levels of control birds (Tonra et al. 2011a). Thus it is possible that hyperphagia and fuel deposition are not directly linked to T, but rather these migratory traits were expressed early due to the early stimulation of the migratory condition resulting from elevated T (see Ramenofsky and Németh 2014).

Generally stopover probability and length of stay are strongly influenced by age, sex, and stopover performance (Moore and Kerlinger 1987; Morris et al. 1994; Morris 1996; Morris et al. 1996; Covino and Holberton 2011; Smolinsky et al. 2013; Covino et al. 2014). Given the lack of relationship between T and foraging rates and fuel deposition rate, it is not surprising that I was also unable
to detect a link between T and stopover likelihood or the length of stay. While longer stopover duration in female BAWW is similar to previous findings of spring migrants (e.g. Morris et al. 1994), it is also interesting to note that in this study male BAWW have higher T levels than females which is typical of other songbird species (see Ketterson et al. 2005). Additionally, while I used minimum stopover length in this study, this measure generally underestimates actual stopover duration (Schaub et al. 2001; Morris et al. 2005) which may have affected my ability to more accurately investigate the influence of T on this aspect of migration biology. Thus, while there was no direct relationship between T and minimum stopover length in BAWW in this study, it may still be possible that T and/or the extent of breeding preparation play a role in how quickly individuals migrate and thus their stopover duration. Males in this and other studies appear to be on a more advanced schedule for breeding preparation (e.g. Covino et al. 2015; Chapters II-IV), and generally male migrants have been shown to migrate faster with shorter stopovers and earlier arrivals on the breeding grounds compared to their female counterparts (see Newton 2008). To more completely investigate the potential influence T has on stopover duration, further studies should employ mark-recapture models (e.g. Schaub et al. 2001; Schaub et al. 2008).

Contrary to my expectation, T levels were lower in recaptured individuals compared to T levels at first capture. Due to sampling constraints, I could only re-sample birds if they were recaptured 48 hours or more after their original capture
and sampling. Many individuals did not stay more than one day at my study site and, of those who stayed, their minimum stopover length was only 2 to 3 days long. These limitations resulted in a low number of re-sampled individuals, dominated by females, and may have undermined accurate representation of variation in T during stopover for this species. That said, T levels have been shown to increase during periods of physical activity in humans (Kuoppasalmi et al. 1976; Zmuda et al. 1996; Sgrò et al. 2014) and other vertebrates (Hu et al. 1998) possibly to promote erythropoiesis or to aid with skeletal muscle anabolism (Terjung 1977). While I am not aware of any studies linking prolonged periods of exercise (e.g. migratory flights) to increased T in birds, my data are consistent with that possibility. Flight tunnel (or wind-tunnel) studies have investigated the effects of flight on other hormones (e.g. Falsone et al. 2009; Jenni-Eiermann et al. 2009) and could address the effects of flight on T.

In this study I did not find any direct correlates between T and measures of migration stopover biology (stopover length, fuel deposition rate) or stopover behaviors (foraging rates, competition). Although indirect, T was higher in some groups where I did detect patterns: (1) males have higher T and also have longer stopovers, (2) birds closer to their breeding grounds, vis-à-vis less depleted feather hydrogen values, have higher T and also forage at faster rates. Overall, T levels in BAWW were lower than what is generally observed in breeding songbirds (Ketterson et al. 2005), however, high T levels and the downstream effects mediated by high T may be antagonistic to the expression of traits
necessary for migration. I looked for correlates to naturally occurring levels of T in this study; however, altering the phenotype of migrants through experimental elevation or suppression of T would allow for a more direct test of the effects T may have on the behaviors and biology of *en route* migrants. While several such experimental studies of captive birds have established the possibility that T plays a role during migration (e.g. Tonra et al. 2011a; Owen et al. 2014), no study has investigated these links during active migration in free-living birds.
APPENDIX A - Genetic Sexing Protocol For Passerines

This protocol was initially optimized for Northern Waterthrush and Swainson’s Thrush but has also been tested on Red-eyed Vireo, Magnolia Warbler, Grey-cheeked Thrush, Hermit Thrush, Grey Catbird, and Northern Cardinal.

A. DNA Extraction with Avian Blood (Taken from Qiagen DNAeasy Kit Protocol with modifications)

Note: this basically follows the Qiagen protocol (kit #69506) but I have made some small modifications. Because I use plasma for other work, samples are ~30-80 µl RBCs suspended in a Blood Lysis Buffer that I make (akin to Queen’s Lysis Buffer).

1. Add 200µl PBS into normal micro-centrifuge tubes (~2ml).
2. Add ~5-15µl RBCs and buffer with pipette with cut tip; be cautious when transferring, blood will be ‘sticky’. Cutting the tip helps with the transfer given the “gloppy” nature of the blood-buffer mix.
3. Add 10µl Proteinase K.
4. Add 200µl AL buffer and vortex ~10 sec.
5. Place on heat block (55°C) for 15-20 min. (If clumps are large, vortex thoroughly half way through).
6. Add 200µl of 100% EtOH and vortex for a few secs.
7. Set pipette to ~700µl and transfer the mixture into the spin column (re-pipette if necessary to get the entire sample).

8. Spin 1-2 min., 14,000 rpm. (Hinges facing out every time when spinning)


10. Add **500µl AW1 buffer** and spin 1-2 min., 14,000 rpm.


12. Add **500µl AW2 buffer** and spin 2-3 min., 14,000 rpm.


   Spin again 1-2 min.

14. Transfer spin columns to final micro-centrifuge tubes.

15. Add **150µl AE Elution Buffer** and let sit 4-5 min.

16. Last spin: You may need to spin in two batches to balance out the tubes, b/c of the extra caps going on. Spin 1-2 min, 14,000 rpm.

17. Remove and discard spin column.

18. Freeze extracted DNA at -20°C (or move onto PCR below).

B. Polymerase Chain Reaction to Amplify CHD genes

   I used the P2 (5' - TCTGCATCGCTAAATCCTTT -3') and P8 (5' – CTCCCAAGGATGAGRAAYTG – 3 ') primers for amplification as has been used before (Griffiths et al. 1996, 1998). I got mine from Eurofins MWG Operon (10 nmole concentration). In preparation and to make my primers last longer I dilute
each by aliquoting out 20 µl primer concentrate into a new tube and adding 100 µl AE Buffer (you can adjust this total volume to meet your needs).

1. Using the PCR tubes that your Pure TAQ Ready-To-Go PCR Bead (Amersham Biosciences, Piscataway, NJ; #27-9557-01) come in, label for your samples.

2. To each tube, add 5µl extracted DNA (thawed), 2µl of each primer (diluted), and 16µl nuclease-free water.

3. Vortex thoroughly making sure that the PCR bead did not get stuck along the sides and dissolves properly. You may also need to do a quick spin in a centrifuge to ensure all the reactants are in the bottom of the tube.

4. Run in thermocycler.

**Thermocycler Settings:**

I use a touch-down protocol where the first cycle has an annealing temp of 60°C (90 sec), and then I drop one degree in successive cycles until reaching an annealing temperature of 55°C. Then 20 cycles with annealing at 55°C.
Table A1.

**Stepdown thermocycler settings.**

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<tr>
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Note: I find that it is best to remove the PCR tubes from the thermocycler soon after the cycle is finished. Store PCR product at 4°C until used.

C. Visualization via Gel Electrophoresis

Although I am sometimes able to use a 1.5% agarose gel, I get much better separation of the bands with a 2% gel. Instructions for a 2% gel are below.

*Making the Gel*

1. Prepare your gel casting tray as required by the model that you have and set a 15 sample comb.
2. Mix 1g agarose powder into 50ml TBE Buffer (1x) in an Erlenmeyer flask.
3. Place a small beaker over the top of the flask to prevent too much evaporation (DO NOT SEAL TIGHTLY).
4. Dissolve powder by heating mixture in standard microwave for 120 seconds. Pause every 20-30 seconds to swirl the flask (be careful not to let it bubble over).

5. When swirling the flask and when removing it, use some kind of heat resistant gloves (not lab gloves) as flask will be hot!

6. Take the flask out of the microwave and let it sit for 1-2 minutes (If the liquid is too hot when you pour it, you might warp your casting tray. But don’t wait too long or the gel will begin to solidify).

7. Pour into casting tray and use a spatula to remove any bubbles.

8. Let sit at least 20 minutes.

9. Remove from casting tray and remove comb.

Running the Gel

People can do this different ways, I prefer to add my loading dye to each PCR product aliquot as I use it in a gel rather than adding it to the whole sample.

1. Glove up!

2. Place your gel in an electrophoresis chamber and cover with 1% TBE Buffer.

3. Take a piece of Parafilm and spread it over a PCR tube tray to make little depressions throughout.

4. Add 1μl loading dye to 15 depressions.
5. Add 5μl of a 100bp DNA ladder to your first depression. Reset your pipette to 6μl and mix the dye and ladder together by pipetting up and down.

6. Add sample to appropriate well.

7. Repeat steps 5 & 6 for your 14 samples changing pipette tips in between samples.

8. Place gel box cover on and plug in (remember to set it so the DNA “runs to red”).

9. Cover setup with a cardboard box or otherwise keep the room darkened during the run (prolonged exposure to light can degrade the DNA).

10. Run at ~50mAMP for 90 to 120 minutes, checking every 20-30 minutes. Since the bands are close together, this has to run for quite a while.

11. Stain in an Ethidium Bromide solution (0.5 μl/ml 1% TBE) for 20-40 minutes (GLOVES!!!! This stuff is very bad for you; keep your staining solution in the dark—it should be good for a month or two). [You may be able to use an alternate stain like SYBR Safe, Gel Red, or Gel Green that are less toxic but I haven’t used anything but EtBr—contact me if you find one that works well].

12. Place on UV light board and visualize at around 300nm. Have a camera hood setup to take a photo of your gel.

13. Score gel. Females have two bands since there are different versions of the CHD genes on the Z and W chromosomes. Males have just one.
Hopefully, if all goes well, your final results should look something like

Figure A1 below.

![Figure A1. Photograph of an agarose gel showing two bands for female songbirds and one band for male songbirds.](image)

References used to compile this protocol include (Griffiths et al. 1996; Griffiths et al. 1998).
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 11052210
PROJECT TITLE: Migratory Connectivity and the En Route Migration Strategies of Migratory Birds
PROPOSED PROJECT DATES: 10/01/2011 to 09/30/2014
PROJECT TYPE: Renewal/Continuation of a Previously Approved Project
PRINCIPAL INVESTIGATOR(S): Frank R. Moore, Ph.D.
COLLEGE/DIVISION: College of Science & Technology
DEPARTMENT: Biological Sciences
FUNDING AGENCY/SPONSOR: National Science Foundation, Moore Basic Research, National Geographic Society
IACUC COMMITTEE ACTION: Full Committee Review Approval
PROTOCOL EXPIRATION DATE: 09/30/2014

Jodie M. Jawor, Ph.D.
IACUC Chair
APPENDIX C - Federal Banding Permit 1

United States Department of the Interior
U.S. GEOLOGICAL SURVEY
PATEKENT WILDLIFE RESEARCH CENTER
BIRD BANDING LABORATORY
12100 BEECH FOREST ROAD STE-4037
LAUREL, MD 20708-4037
301-497-5790

FEDERAL BIRD BANDING PERMIT

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<td>21221</td>
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<tr>
<td>DEPT BIO SCI, UNIV SOUTHERN MS</td>
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Signature of Issuing Official, Chief, Bird Banding Laboratory

Signature of Permittee

Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

Permittee is Authorized To Band:

- All Species, Except Waterfowl, Eagles or Endangered Threatened Species Unless Specified

In the States of:

- AI * AK * AZ * CA * FL * LA * MA * MI * MS * NH * NY * RI * UT *

With Special Authorization to:

- Use USGS Bands On Above Listed North American Migrators
- Upon Host County Approval
- Band
- Take, possess and transport blood samples not to exceed 1½ body mass
- Take, possess and transport feather samples
- Use Misc nets
- Trap

And Additionally Authorized To Use The Following Auxiliary Marking Authorization/s:

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| Plastic Color | AMERICAN REDSTART | Black, Blue, Brown | Mobile, AL | 3 | ALSO NAT. BROWN ON FEDERAL BAND |
| Leg Band (01A) | BLACK-AND-WHITE WABLER | Green, Mauve | Baldwin, AL | | |
| | GRAY CATBIRD | Orange, Pink, Purple | Cameron, LA | | |
| | MAGNOLIA WABLER | Red, White, Yellow | | | |

| Plastic Color | CHUCK-WILL'S-WIDOW | Black, Blue, Brown | Baldwin, AL | 7 | NTE 3% TOTAL BODYT WT: 16-31/7 MHZ: |
| Leg Band (01A) | GRAY CATBIRD | Green | Vernon, LA | | |
| | (other) | Red | | | |
### Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization(s) listed below:

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</table>
Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below.

The following Subpermittee/s are authorized to band under the direction of the above permittee, in accordance with the same general conditions, and the subpermittee specific authorizations listed below:

(No. of Active Sub Permittees: 3)

<table>
<thead>
<tr>
<th>Permit Number</th>
<th>Action</th>
<th>Action Date</th>
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<th>Valid Until</th>
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<tr>
<td>21221</td>
<td>Revise</td>
<td>12/16/79</td>
<td>11/23/79</td>
<td>07/31/81</td>
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</tbody>
</table>

**21221 - M**

**MS EMILY COHEN**

DEPT BIO SCI, UNIV SOUTHERN MS BOX 5018
HATTIESBURG, MS 39406 5018

Is Authorized To Band:
Passerines and Near-passerines except Endangered/Threatened Species Unless Specified

In the States Of:
LA * MS *

With Special Authorization to:
Band
Auxiliary mark
Use Mist nets
Trap

---

**21221 - N**

**MS KRISTINA PAXTON**

DEPT BIO SCI, UNIV SOUTHERN MS BOX 118 COLLEGE
DRIVE HATTIESBURG, MS 39406 0001

Is Authorized To Band:
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified

In the States Of:
AL * AK * AZ * CA * LA * MS *

With Special Authorization to:
Band
Auxiliary mark
Use Mist nets
Trap

---

**21221 - O**

**MS JACLYN SMOLINSKY**

DEPT BIO SCI, UNIV SOUTHERN MS BOX 5018
HATTIESBURG, MS 39406 5001

Is Authorized To Band:
Passerines and Near-passerines except Endangered/Threatened Species Unless Specified

In the States Of:
AL * LA * MS *

With Special Authorization to:
Band
Auxiliary mark
Use Mist nets
Trap
Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

21221 - P  
THEODORE ZENZAL  
DEPARTMENT OF BIOLOGICAL SCIENCES  
THE UNIV OF  
SOUTHERN MISSISSIPPI  
HATTIESBURG, MS 39406

Is Authorized To Band:
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified
In the States Of:
AL  *  LA  *  MS  *

With Special Authorization to:
Band
Auxiliary mark
Use Mist nets
Trap

21221 - Q  
MC JILL GAUTHERAUX  
DEPT BIO SCI, UNIV SOUTHERN MS 119 COLLEGE  
DRIVE HATTIESBURG, MS 39406-0001

Is Authorized To Band:
Passerines and Near-passerines except Endangered/Threatened Species Unless Specified
In the States Of:
AL  *  LA  *  MS  *

With Special Authorization to:
Band
Take, possess and transport blood samples-not to exceed 1% body mass
Use Mist nets
Trap
Comments:
COLLECT FEATHER SAMPLES FOR BRUCE PETERJOHN 03/07/2011

21221 - R  
MC KRISTEN COVINO  
DEPT BIO SCI, UNIV SOUTHERN MS 119 COLLEGE  
DRIVE HATTIESBURG, MS 39406-0001

Is Authorized To Band:
Passerines and Near-passerines except Endangered/Threatened Species Unless Specified
In the States Of:
AL  *  LA  *  MS  *

With Special Authorization to:
Band
Take, possess and transport blood samples-not to exceed 1% body mass
Use Mist nets
Trap
Comments:
COLLECT FEATHER SAMPLES FOR BRUCE PETERJOHN 03/07/2011
<table>
<thead>
<tr>
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<th>Personal</th>
<th>Permit Number</th>
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<tbody>
<tr>
<td>DR FRANK R. MOORE</td>
<td>DEPT BIOSCI, UNIV SOUTHERN MS BOX 5018 HATTIESBURG, MS 39406 001</td>
<td>21221</td>
<td>Revise</td>
<td>12/10/13</td>
<td>11/23/79</td>
<td>07/31/19</td>
</tr>
</tbody>
</table>

Signature of Issuing Official, Chief, Bird Banding Laboratory: [Signature]

Signature of Permittee: [Signature]

Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

21221 - 8

MR WILLIAM H LEWIS 118 COLLEGE DR BOX 5018 UNIV OF SOUTHERN MISSISSIPPI HATTIESBURG, MS 37456

Is Authorized To Band:

- Passerines and Near-passerines except Endangered/Threatened Species Unless Specified
- In the States Of:
- LA *

With Special Authorization to:

- Band
- Take, possess and transport blood samples not to exceed 1% body mass
- Take, possess and transport feather samples
- Use Mist nets
FEDERAL BIRD BANDING PERMIT

Under the provisions of Regulations issued under the Migratory Bird Treaty Act of July 3, 1918 (60 Stat. 755) as amended, or the Bald Eagle Act of June 8, 1940 (54 Stat. 235) as amended, the person named herein is authorized to capture, for scientific banding or marking purposes, those migratory birds described hereon and to salvage birds accidentally killed during normal banding activities.

This permit is subject to the terms, exceptions and restrictions expressed herein or on the reverse side hereof and is further subject to any applicable Federal, State, Tribal or Federal Regulations.

This permit is invalid unless accompanied by any required State permits or licenses.

GENERAL CONDITIONS

1. The Permittee is not authorized to capture or possess migratory birds for any reason other than banding, marking or salvage of banding mortalities for scientific purposes. NOW THIS PERMITTEE ALLOWED TO HOLD MIGRATORY BIRDS FOR A PERIOD OF MORE THAN 24 HOURS. Live birds shall be released as soon as practical after capture.

2. You may donate dead migratory birds or any part thereof (except banded and tagged eagles, and species listed as threatened and endangered) without additional authorization from the migratory bird permit issuing office to public institutions (as specified in 50 CFR 10.13) or individuals or entities authorized by permit to acquire and possess migratory bird specimens for educational purposes. All dead specimens that you do not transfer to another authorized party must be disposed of by such means as are necessary to ensure that they are not exposed to animals in the wild.

3. You may not salvage and must immediately report to the USFWS Office of Law Enforcement any dead or injured migratory birds that you encounter that appear to have been poisioned, shot, electrocuted, collided with industrial power generation equipment, or were otherwise killed or injured as the result of potential criminal activity. Please contact BRI for more information.

4. All eagle feathers and/or whole eagle carcasses must be shipped to the National Eagle Repository. Contact: U.S. Fish and Wildlife Service, National Eagle and Wildlife Repository, 5550 Iowan St., RMA Building 125, Commerce City, Colorado 80642, (303) 217-2110.

5. The Permittee shall keep RECORDS accounting for the use of all bands received. Periodic RECORDS COVERING THE USE OF THESE BANDS shall be submitted to the Bird Banding Laboratory in accordance with the instructions received therefrom. Failure to provide data in accordance with the instructions received from the Bird Banding Laboratory is sufficient justification for the revocation of this permit. The Permittee shall keep records of disposition of salvaged banding mortalities for a period of five years and shall be reported to the Bird Banding Laboratory upon request.

6. The holder of this permit shall not sell, exchange, or transfer bands to unauthorized bands or to the general public. All transfers to authorized bands must be communicated to the Bird Banding Laboratory prior to the transfer of bands. Any unused bands remaining when this permit is voluntarily returned, revoked, or expired must be returned to the Bird Banding Laboratory.

7. The Permittee shall, at all reasonable hours, allow any authorized representative of the U.S. Geological Survey or the U.S. Fish and Wildlife Service to ENTER and INSPECT the premises where operations authorized by this permit are being conducted and shall allow such representative to inspect the records relating to such operations.

8. This permit may be SUSPENDED or REVOKED by the Director of the U.S. Geological Survey or authorized representative, if the Permittee violates any of the provisions of the regulations under which this permit is issued or if the Permittee fails to render promptly any reports required.

This permit is, at all times, subject to suspension or revocation at the discretion of the Director or representative.

9. This permit is not transferable and must be in possession of the Permittee when exercising the authorities granted herein.

10. All traps, nets or other capture devices shall bear a TAG or LABEL showing the name, address and permit number of the Permittee; alternatively the trapping area shall be adequately marked with POSTERS provided by the Bird Banding Laboratory. The Permittee's name, address and permit number shall be legibly displayed on such posters.

11. This permit DOES NOT authorize the capture of any birds on any property, public or private without the CONSENT OF THE OWNER OR CUSTODIAN THEREOF.

12. All banding under this permit is in accordance with the principles, spirit, and intent of the Animal Welfare Act of 1976 and the most recent revision of the Ornithological Council’s Guidance on the Use of Wild Birds in Research.

13. Unless specifically noted on the reverse, the following ARE NOT AUTHORIZED:
   a. The taking of blood or feather sampling from any bird.
   b. The use of ANY BAND, strap, paint, dye, signal-sending device or any marking device other than the official numbered leg bands issued by the Bird Banding Laboratory.
   c. The use of MIG. NETS or other net for the catching of birds.
   d. The use of TRANQUILIZING DRUGS OR OTHER CHEMICALS for the purpose of capturing birds.
   e. Trapping or disturbing the nests or nestlings, for the purpose of banding or marking, of species designated by the Secretary of Interior as “ENDANGERED” or “THREATENED”.
   f. The handling of any PREVIOUSLY BANDED BIRD in any manner which may bias data or sample in the Bird Banding Laboratory which pertain to that bird or which may alter that bird’s survival potential, behavior or other normal characteristics. This activity includes adding markers to or removing markers from previously banded birds.

FORM 5-473
(AUG. 2011)
APPENDIX D - Federal Banding Permit 2

United States Department of the Interior
U.S. GEOLOGICAL SURVEY
PATEKENT WILDLIFE RESEARCH CENTER
BIRD BANDING LABORATORY
12100 BEECH FOREST ROAD STE-4037
LAUREL, MD 20708-4037
301-497-5790

FEDERAL BIRD BANDING PERMIT

Permittee:  DR. SARA R. MORRIS
Deft Bsc. CANISIUS COLLEGE
231 MAIN STREET
BUFFALO, NY 14208 1098

Permit Number:  22241
Action:  Renew
Action Date:  07/10/12
Issue Date:  11/19/87
Valid Until:  07/31/15

Signature of Issuing Official, Chief, Bird Banning Laboratory

Signature of Permittee

Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below.

Permittee is Authorized To Band:

All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified Hummingbirds

In the States of:

ME * MD * MA * NJ * NY * VT *

With Special Authorization to:

Band
Take, possess and transport blood samples—not to exceed 1% body mass
Take, possess and transport feather samples
Use Mist nets
Trap

And Additionally Authorized to Use The Following Auxiliary Marking Authorization/s:

<table>
<thead>
<tr>
<th>Marker Type</th>
<th>Species</th>
<th>Colors</th>
<th>Locations</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>BARN SWALLOW BLACK-CAPPED</td>
<td>Black, Blue,</td>
<td>York, ME</td>
<td>APPLEDORE IS.; USE CAUTION WITH COLOR BANDS ON SWALLOWS; THE TARSUS MAY BE TOO SHORT FOR TWO BANDS/LEG</td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>BLACK-CAPPED CHICKADEE</td>
<td>Green, Orange, Pink, Purple,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>COMMON YELLOWthroat GRAY CATBIRD</td>
<td>Red, White, Yellow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>DOWY WOODPIECKER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>NORTHERN CARDINAL RED-NESTED</td>
<td></td>
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</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>RED-NESTED NUTHATCH</td>
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<td></td>
<td></td>
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<tr>
<td>01A Plastic Color Leg Band</td>
<td>RED-WINGED BLACKBIRD ROSE-NESTED GROSBEAK</td>
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<td></td>
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</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>TUFTED TITMSONE WHITE-NESTED NUTHATCH</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>GREAT BLACK-BACKED GULL HERRING GULL</td>
<td>Black, Blue, ME</td>
<td></td>
<td>APPLEDORE IS. NO CODES ON BANDS</td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td></td>
<td>Green, Orange, Pink, Red, White, Yellow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>01A Plastic Color Leg Band</td>
<td>HERRING GULL</td>
<td>Green</td>
<td>ME</td>
<td>ALPHA-NUMBER-NUMBER CODED WITH ALPHA-A, C, E, F, H, J, K</td>
</tr>
</tbody>
</table>
Permittee:  
DR SARA R MORRIS  

DEPT BIO  CANISIUS COLLEGE  
1011 MAIN STREET  
BUFFALO, NY 14208 1098  

Permit Number: 22242  
Action: Renew  
Action Date: 07/10/12  
Issue Date: 11/10/67  
Valid Until: 07/21/15  

Signature of Issuing Official,  
Chief, Bird Banding Laboratory: Bruce Petersen  

Signature of Permittee:  

Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization(s) listed below:

<table>
<thead>
<tr>
<th>Marker Type</th>
<th>Species</th>
<th>Colors</th>
<th>Locations</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>09 Transmitter</td>
<td>GREAT BLACK-BACKED</td>
<td>Black</td>
<td>York, ME</td>
<td>White codes, blue, pink, purple, red, white, yellow</td>
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<tr>
<td></td>
<td>CHILL</td>
<td></td>
<td></td>
<td>NTE 36 local body weight, rapples-style harness. Please advise of Msa range.</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>01A Plastic</td>
<td>ROSENECK WHEN</td>
<td>Black, Blue,</td>
<td>Niagara, NY</td>
<td></td>
</tr>
<tr>
<td>Color Leg</td>
<td></td>
<td></td>
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<tr>
<td>Band</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

The following Subpermittee(s) are authorized to band under the direction of the above permittee, in accordance with the same general conditions, and the subpermittee specific authorizations listed below:  

(End of Number Of Active Subpermittees 15)

**22243 - A**  
HOLMES  D W  
5643A HARPER'S FARM ROAD  COLUMBIA, MD 21044  

Is Authorized To Band:  
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified In the States Of:  
ME * MD *  

With Special Authorization to:  
Band  
Auxiliary mark  
Take, possess and transport feather samples  
Use Mist nets  
Trap
Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization(s) listed below:

<table>
<thead>
<tr>
<th>Permit Number</th>
<th>Action</th>
<th>Action Date</th>
<th>Issue Date</th>
<th>Valid Until</th>
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<tbody>
<tr>
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<tr>
<td>22243 - R</td>
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</tr>
<tr>
<td>22243 - P</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Is Authorized To Band:

All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified In the States Of:

#### ME *

With Special Authorization to:

- Band
- Auxiliary mark
- Take, possess and transport feather samples
- Use Mist nets
- Trap

#### NH *

With Special Authorization to:

- Band
- Auxiliary mark
- Take, possess and transport feather samples
- Use Mist nets
- Trap

#### NJ *

With Special Authorization to:

- Band
- Auxiliary mark
- Take, possess and transport feather samples
- Use Mist nets
- Trap
Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

<table>
<thead>
<tr>
<th>Permit Number</th>
<th>Action</th>
<th>Action Date</th>
<th>Issue Date</th>
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<tr>
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<td>Renew</td>
<td>07/10/12</td>
<td>11/19/87</td>
<td>07/31/15</td>
</tr>
</tbody>
</table>

**22243 - C**

**Is Authorized To Band:**
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified

**In the States Of:**
- ME *
- NH *

**With Special Authorization to:**
Band
Auxiliary mark
Take, possess and transport feather samples
Use Mist nets
Trap

**22243 - R**

**Is Authorized To Band:**
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified

**In the States Of:**
- ME *
- MA *
- NH *

**With Special Authorization to:**
Band
Auxiliary mark
Take, possess and transport feather samples
Use Mist nets
Trap

**22243 - T**

**Is Authorized To Band:**
GREAT BLACK-BACKED GULL
HEERING GULL
LESSER BLACK-BACKED GULL

**In the States Of:**
- ME *
- NH *

**With Special Authorization to:**
Band
Auxiliary mark
Take, possess and transport blood samples-not to exceed 1% body mass
Take, possess and transport feather samples
Trap
Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

**22243 – J**  
**Ms Elizabeth Redtenbacher**  
49 Oyster River Road  
Exeter, NH 03833

**Is Authorized To Band:**
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified

**In the States Of:**
ME * NH *

**With Special Authorization to:**
Band
Auxiliary mark
Take, possess and transport feather samples
Use mist nets
Trap

**22243 – X**  
**Mr David Bent**  
Cornell Lab of Ornithology 159 Sapsucker
Wood Road Ithaca, NY 14850

**Is Authorized To Band:**
All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified

**In the States Of:**
ME * NH *

**With Special Authorization to:**
Band
Auxiliary mark
Take, possess and transport blood samples—not to exceed 1% body mass
Take, possess and transport feather samples
Use mist nets
Trap

**22243 – L**  
**Ms Lindsey Walters**  
Canisius College 2001 Main Street  
Buffalo, NY 14208

**Is Authorized To Band:**

**In the States Of:**
NY *

**With Special Authorization to:**
Band
Auxiliary mark
Take, possess and transport blood samples—not to exceed 1% body mass
Take, possess and transport feather samples
Use mist nets
Trap
Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below.

<table>
<thead>
<tr>
<th>Permit Number</th>
<th>Action</th>
<th>Action Date</th>
<th>Issue Date</th>
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<td>Renew</td>
<td>07/10/12</td>
<td>11/19/87</td>
<td>07/31/15</td>
</tr>
</tbody>
</table>

### 22243 - M

**Permittee:**

Name: KRISTEN COVINO  
Address: 27 WOOD STREET, APT. 1  OLD TOWN, ME 04462

**Authorized To Band:**

- All Species Except Waterfowl, Eagles or Endangered/Threatened Species Unless Specified

**In the States Of:**

- ME  
- NH

**With Special Authorization to:**

- Band
- Auxiliary mark
- Take, possess and transport blood samples-not to exceed 1% body mass
- Take, possess and transport feather samples
- Use Mist nets
- Trap

### 22243 - M

**Permittee:**

Name: MATTHEW LEVIVA  
Address: 187 CONSTANT AVE  STATEN ISLAND, NY 10314

**Authorized To Band:**

- BARN SWALLOW
- TREE SWALLOW

**In the States Of:**

- ME

**With Special Authorization to:**

- Band
- Auxiliary mark
- Take, possess and transport blood samples-not to exceed 1% body mass
- Take, possess and transport feather samples
- Use Mist nets
- Trap

### 22243 - O

**Permittee:**

Name: NICOLAS LOUIS  
Address: 8 SHORETACE DRIVE  MARSHALL, NY 10933

**Authorized To Band:**

- BARN SWALLOW
- TREE SWALLOW

**In the States Of:**

- ME

**With Special Authorization to:**

- Band
- Auxiliary mark
- Take, possess and transport blood samples-not to exceed 1% body mass
- Take, possess and transport feather samples
- Use Mist nets
- Trap
<table>
<thead>
<tr>
<th>Permit Number</th>
<th>Action</th>
<th>Action Date</th>
<th>Issue Date</th>
<th>Valid Until</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Renew</td>
<td>07/10/12</td>
<td>11/19/87</td>
<td>07/31/15</td>
</tr>
</tbody>
</table>

**Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:**

### 22243 - P
**Is Authorized To Band:**
- Tree Swallow

**In the States Of:**
- ME

**With Special Authorization to:**
- Band
- Auxiliary mark
- Trap

**Permittee:**
- MR KYLE HUTTON
- 400 TINKHAM ROAD
- ATTICA, NY 14001

### 22243 - Q
**Is Authorized To Band:**
- Passerines and Near-passerines

**In the States Of:**
- ME, NY, VT

**With Special Authorization to:**
- Band
- Take, possess and transport blood samples-not to exceed 1% body mass
- Use Mist nets
- Trap

**Permittee:**
- LINDSAY BISCH, SERBY
- 1 BRENMAN CIRCLE, BOX 302
- DOOLIN, VT 05726
FEDERAL BIRD BANDING PERMIT

Under the provisions of Regulations issued under the Migratory Bird Treaty Act of July 3, 1918 (60 Stat. 755) as amended, or the Bald Eagle Act of June 5, 1940 (54 Stat. 235) as amended, the person named hereon is authorized to capture, for scientific banding or marking purposes, those migratory birds described hereon and to salvage birds killed or injured during normal banding activities.

This permit is subject to the terms, exceptions and restrictions expressed herein or on the reverse side hereof and is further subject to any applicable Territorial, State, Tribal or Federal Regulations.

This permit is invalid unless accompanied by any required State permits or licenses.

GENERAL CONDITIONS

1. The Permittee is not authorized to capture or possess migratory birds for any reason other than banding, marking or salvage of banding mortalities for scientific purposes. NOTE: THIS PERMITTEE ALLOWED TO HOLD MIGRATORY BIRDS FOR A PERIOD OF MORE THAN 24 HOURS. Live birds shall be released as soon as practical after capture.

2. You may donate dead migratory birds or any part thereof (except lead shot and golden eagles and species listed as threatened and endangered) without additional authorization from the migratory bird permit issuing office to public institutions (as specified in 50 CFR 17.13) or individuals or entities authorized by permit to acquire and possess migratory bird specimens for educational purposes. All dead specimens that you do not transfer to another authorized party must be disposed of by such means as are necessary to ensure that they are not exposed to animals in the wild.

3. You may not salvage and must immediately report to the USFWS Office of Law Enforcement any dead or injured migratory birds that you encounter that appear to have been poisoned, shot, electrocuted, or otherwise killed or injured as the result of potential criminal activity. Please contact FBI for more information.

4. All eagle feathers and/or whole eagle carcasses must be shipped to the National Eagle Repository. Contact: U.S. Fish and Wildlife Service, National Eagle and Wildlife Repository, 5550 Havasu St., RMA Building 125, Commerce City, Colorado 80022, (303) 217-2110.

5. The Permittee shall keep RECORDS accounting for the use of all bands received. Periodic RECORDS COVERING THE USE OF THESE BANDS shall be submitted to the Bird Banding Laboratory in accordance with the instructions received therefrom. Failure to provide data in accordance with the instructions received from the Bird Banding Laboratory is sufficient justification for the revocation of this permit. The Permittee shall keep records of disposition of salvaged banding mortalities for a period of five years and shall be reported to the Bird Banding Laboratory upon request.

6. The holder of this permit shall not sell, exchange, or transfer bands to unauthorized bands or to the general public. All transfers to unauthorized bands must be communicated to the Bird Banding Laboratory prior to the transfer of bands. Any unused bands remaining when this permit is voluntarily returned, revoked, or expired must be returned to the Bird Banding Laboratory.

7. The Permittee shall, at all reasonable hours, allow any authorized representative of the U.S. Geological Survey or the U.S. Fish and Wildlife Service to ENTER and INSPECT the premises where operations authorized by this permit are being conducted and shall allow such representative to inspect the records relating to such operations.

8. This permit may be SUSPENDED or REVOKED by the Director of the U.S. Geological Survey or authorized representative, if the Permittee violates any of the provisions in the regulations under which this permit is issued or if the Permittee fails to render promptly any reports required. This permit, at all times, subject to suspension or revocation at the discretion of the Director or representative.

9. This permit is not transferable and must be in possession of the Permittee when exercising the authorizations granted herein.

10. All traps, nets or other capture devices shall be a TAG or LABEL showing the name, address and permit number of the Permittee, alternatively the trapping area shall be adequately marked with POSTERS provided by the Bird Banding Laboratory. The Permittee's name, address and permit number shall be legibly displayed on such posters.

11. This permit DOES NOT authorize the capture of any birds on any property, public or private without the CONSENT OF THE OWNER OR CUSTODIAN THEREOF.

12. All banding under this permit is in accordance with the principles, spirit, and intent of the Animal Welfare Act of 1970 and the most recent revision of the Ornithologists' Council's Guidelines in the Use of Wild Birds in Research.

13. Unless specifically noted on the reverse, the following ARE NOT AUTHORIZED:
   a. The taking of blood or feather sampling from any bird.
   b. The use of ANY BAND, clip, paint, dye, signal-sending device or any marking device other than the official numbered leg bands issued by the bird banding laboratory.
   c. The use of MIGR NETS or other nets for the capturing of birds.
   d. The use of TRANQUILIZING DRUGS or OTHER CHEMICALS for the purpose of capturing birds.
   e. Trapping or disturbing the nests or nestlings, for the purpose of banding or marking, of species designated by the Secretary of Interior as "ENDANGERED" or "THREATENED.
   f. The handling of any PREVIOUSLY BANDED BIRD in any manner which may bias data on the bird banding laboratory which pertain to that bird or which may alter that bird's survival potential, behavior or other normal characteristics. This specificity includes altering markers to or removing markers from previously banded birds.

Form B-473
(August 2011)
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