Ketterson / Nolan Research Group Collection

This document is part of a collection that serves two purposes. First it is a public archive for data and documents resulting from evolutionary, ecological, and behavioral research conducted by the Ketterson-Nolan research group. The focus of the research is an abundant North American songbird, the dark-eyed junco, *Junco hyemalis*, and the primary sources of support have been the National Science Foundation and Indiana University. The research was conducted in collaboration with numerous colleagues and students, and the objective of this site is to preserve not only the published products of the research, but also to document the organization and people that led to the published findings. Second it is a repository for the works of Val Nolan Jr., who studied songbirds in addition to the junco: in particular the prairie warbler, *Dendroica discolor*. This site was originally compiled and organized by Eric Snajdr, Nicole Gerlach, and Ellen Ketterson.

Context Statement
This document was generated as part of a long-term biological research project on a songbird, the dark-eyed junco, conducted by the Ketterson/Nolan research group at Indiana University. For more information, please see IUScholarWorks (https://scholarworks.iu.edu/dspace/handle/2022/7911).

Annual Report for Period: 08/2010 - 07/2011
Submitted on: 11/24/2011
Principal Investigator: Ketterson, Ellen D.
Award ID: 0820055
Organization: Indiana University
Submitted By:
Ketterson, Ellen - Principal Investigator
Title:
Hormones and Phenotypic Integration: Comparing Sexes, Individuals and Populations

Project Participants

Senior Personnel

Name: Ketterson, Ellen
Worked for more than 160 Hours: Yes
Contribution to Project:
Ketterson is the PI and lead investigator on this project. She participates in field and lab work and manages the research team.

Post-doc

Name: Whittaker, Danielle
Worked for more than 160 Hours: Yes
Contribution to Project:
Research Associate with molecular skills. Dr. Whittaker is lab organizer and in charge of pheromone project in juncos. Supported by Indiana University

Name: Rosvall, Kim
Worked for more than 160 Hours: Yes
Contribution to Project:
NRSA postdoctoral research trainee. Dr. Rosvall is studying aggression in female juncos and brain sensitivity to testosterone. Supported by NIH training grant

Graduate Student

Name: O'Neal, Dawn
Worked for more than 160 Hours: Yes
Contribution to Project:
Graduate student who studied impact of experimental elevation of T on female parental behavior in juncos. Former NSF GRF, currently supported by NIH on training grant

Name: Cain, Kristal
Worked for more than 160 Hours: Yes
Contribution to Project:
Graduate student who is studying female behavioral syndromes and androgyny in juncos. NSF GRF fellow currently supported by NIH training grant

Name: Reichard, Dustin
Worked for more than 160 Hours: Yes
Contribution to Project:
Graduate student who is studying vocal behavior and speciation in the junco. NSF GRF fellow who is currently supported by Indiana University

Name: Atwell, jonathan
Worked for more than 160 Hours: Yes
Contribution to Project:

Graduate student who is studying hormonal and behavioral differences between an urban and a native population of juncos in California. NSF GRF supported by NSF.

Name: Peterson, Mark

Worked for more than 160 Hours: Yes

Contribution to Project:

Graduate student who is studying genetic variation in juncos (transcriptomics). He is supported by Indiana University

Name: Bergeon, Christine

Worked for more than 160 Hours: Yes

Contribution to Project:

Graduate student who is studyng hormones and behavioral variation in a South Dakota population of juncos. NSF GRF supported by NSF.

Name: Gerlach, Nicole

Worked for more than 160 Hours: Yes

Contribution to Project:

Graduate student who is studying sexual selection and multiple paternity in juncos. Former NSF GRF currently supported by Indiana University

Name: Hanauer, Rachel

Worked for more than 160 Hours: Yes

Contribution to Project:

Beginning graduate student, paid hourly, learning techniques in field and laboratory

Name: Carlton, Liz

Worked for more than 160 Hours: Yes

Contribution to Project:

Conducted field research on immune function in Virginia, supported by Indiana University

Undergraduate Student

Name: Richmond, Kaitlin

Worked for more than 160 Hours: Yes

Contribution to Project:

Lab research on bird odors in 2010, volunteer intern in Indiana, in charge of captive breeding project in 2011

Name: Swanger, Elizabeth

Worked for more than 160 Hours: Yes

Contribution to Project:

Assistant in Indiana, captive birds and data analysis, paid hourly

Name: Welkin, Joe

Worked for more than 160 Hours: Yes

Contribution to Project:

Joe enrolled for undergraduate research hours to study vocal behavior in the junco

Technician, Programmer

Name: Kiley, Ryan

Worked for more than 160 Hours: Yes

Contribution to Project:

Lab Organizer, Animal Caretaker, Research Associate supported by this award

Name: Dapper, Amy

Worked for more than 160 Hours: Yes

Contribution to Project:

Research Associate who managed data, organized field season, and conducted field research. Supported by NSF

Name: Wanamaker, Sarah

Worked for more than 160 Hours: Yes

Contribution to Project:

Post undergraduate student who conducted field research on the junco in Virgina. Supported by this award from NSF for two summers, then hired full-time to manage animal facility and data and to help with compliance

Name: Burns, Steve

Worked for more than 160 Hours: Yes

Contribution to Project:

Steve Burns is a documentary film maker and he is working on a broader impacts project to bring junco research to the public

Other Participant

Name: Schultz, Elizabeth

Worked for more than 160 Hours: Yes

Contribution to Project:

Post-undergraduate/pre-graduate student studying hormones and behavior of juncos in South Dakota. Supported by this NSF award

Name: Shreve, Megan

Worked for more than 160 Hours: Yes

Contribution to Project:

Megan was a field assistant at Mountain Lake Biological Station. She is headed for graduate school and wanted the experience

Name: Drouley, Marine

Worked for more than 160 Hours: Yes

Contribution to Project:

Marine Drouilly was a field assistant on the junco project in Wyoming

Research Experience for Undergraduates

Name: Chaby, Lauren

Worked for more than 160 Hours: Yes

Contribution to Project:

REU undergraduate student who studied female parental behavior in the junco. Supported by REU site award to University of Virginia

Years of schooling completed: Junior

Home Institution: Other than Research Site

Home Institution if Other: Clarkson University

Home Institution Highest Degree Granted(in fields supported by NSF): Bachelor's Degree

Fiscal year(s) REU Participant supported: 2009

REU Funding: REU supplement

Name: Rich, Miriam

Worked for more than 160 Hours: Yes

Contribution to Project:

REU undergraduate who studied aggressive behavior in female juncos. Supported by REU site award to University of Virginia

Years of schooling completed: Junior

Home Institution: Other than Research Site

Home Institution if Other: Swarthmore College

Home Institution Highest Degree Granted(in fields supported by NSF): Bachelor's Degree

Fiscal year(s) REU Participant supported: 2009

REU Funding: REU supplement

Name: Rice, Rebecca

Worked for more than 160 Hours: Yes

Contribution to Project:

Undergraduate research student during the academic year comparing populations of juncos for aggresssion, REU undergraduate student studying vocal behavior in juncos. Supported by Indiana University and by an REU site award to the University of Virginia

Years of schooling completed: Freshman

Home Institution: Other than Research Site

Home Institution if Other: Indiana University

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2009

REU Funding: REU supplement

Name: Boser, Matt

Worked for more than 160 Hours: Yes

Contribution to Project:

REU researcher studying hormones and behavior in juncos in South Dakota.
Supported by REU supplement to this award

Years of schooling completed: Junior

Home Institution: Other than Research Site

Home Institution if Other: Eastern Connecticut University

Home Institution Highest Degree Granted(in fields supported by NSF): Master's Degree

Fiscal year(s) REU Participant supported: 2009

REU Funding: REU supplement

Name: Tonge, Natasha

Worked for more than 160 Hours: Yes

Contribution to Project:

Field and laboratory work on aggression and transcripion of aromatase enzyme, REU supplement

Years of schooling completed: Junior

Home Institution: Same as Research Site

Home Institution if Other: Swarthmore Collleg

Home Institution Highest Degree Granted(in fields supported by NSF): Bachelor's Degree

Fiscal year(s) REU Participant supported: 2010

REU Funding: REU supplement

Name: Vanderbilt, Carla

Worked for more than 160 Hours: Yes

Contribution to Project:

Field research on bird song, REU student in U of Virginia's program at Mountain Lake Biological Station

Years of schooling completed: Junior

Home Institution: Same as Research Site

Home Institution if Other: Stetson University

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2010

REU Funding: REU supplement

Name: Pandit, Meelyn

Worked for more than 160 Hours: Yes

Contribution to Project:

Field research on bird song, REU student in U of Virginia's program at Mountain Lake Biological Station

Years of schooling completed: Freshman

Home Institution: Same as Research Site

Home Institution if Other: Indiana University

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2010

REU Funding: REU supplement

Name: Fetters, Tamara

Worked for more than 160 Hours: Yes

Contribution to Project:

Field research on avian odors, REU student in U of Virginia's program at Mountain Lake Biological Station

Years of schooling completed: Junior

Home Institution: Same as Research Site

Home Institution if Other: Virginia Tech University

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2010

REU Funding: REU supplement

Name: Squibb, Cari Lynn

Worked for more than 160 Hours: Yes

Contribution to Project:

REU student at Mountain Lake Biological Station supported by award to MLBS and working with graduate student Rachel Hanauer or immunoecology

Name: Jayaratna, Sonya

Worked for more than 160 Hours: Yes

Contribution to Project:

Sonya was an REU student in the REU site award to the Center for the Integrative Study of Animal Behavior. She studied individual variation in m-RNA expression for androgen receptor in juncos using Q-PCR

Years of schooling completed: Freshman

Home Institution: Same as Research Site

Home Institution if Other:

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2011

REU Funding: REU site award

Name: Fergusen, Stephen

Worked for more than 160 Hours: Yes

Contribution to Project:

Stephen was supported on an REU supplement to this award. He spent time in the field at Mountain Lake Biological Station and in Wyoming studying hormonal responses to song playbacks in the junco. He attended the ANimal Behavior Society meeting at the end of the summer.

Organizational Partners

University of Virginia Main Campus

One of field sites is Mountain Lake Biologica Station which belongs to the University of Virginia

Other Collaborators or Contacts

Barney Schlinger, UCLA
John C. Wingfield, UCDavis
Tom Hahn, UC Davis
Trevor Price, U of Chicago
Jim Goodson, Indiana U
Dale Sengelaub, Indiana U
Greg Demas, Indiana University
Borja Mila, Madrid Museum
Goncalo Cardoso, University of Melbourne
John Colbourne, Indiana University
Greg Demas, Indiana University
Dave Enstrom, University of Illinois
Garth Spelman, University of South Dakota
Haixu Tang, Indiana University

Activities and Findings

Research and Education Activities: (See PDF version submitted by PI at the end of the report)

NSF IOB-0820055
Title: Hormones and phenotypic integration: comparing sexes, individuals, and populations, $570,000, 8/01/08-7/31/12
Year 3 report, research and education activities, 8/1/10-7/31/11
[Some of this is a repeat from the year 1 and the year 2 report; new material has also been added]

Research goals, conceptual
Natural selection shapes organisms as integrated sets of traits, but the relative ease with which these traits can be assembled and disassembled in response to selection is contentious. Hormones often underlie the co-expression of traits, and hormonal correlations, like genetic correlations, can promote adaptation or delay evolutionary response. The relative importance of phenotypic integration and independence of hormonally mediated traits has significant implications for the evolution of life histories, sexual dimorphism, and population divergence. Integration and independence can be studied via experimental manipulations of hormonal phenotypes, assessment of patterns of natural variation in hormones in relation to phenotype and fitness, comparisons of hormonal phenotypes across populations, and mechanistic studies of hormones and their interaction with target tissues. The research supported by this award employs all these approaches by focusing on the steroid hormone testosterone and its integrating effect on the phenotype of males and females of a songbird species, the dark-eyed junco.

Research goals, chronological
Since 1986 our research group has studied the integrating effect of the steroid hormone testosterone on behavior and physiology in a songbird. Our subject is the Dark-eyed Junco (Junco hyemalis), and we study free-living and captive individuals from Virginia, Indiana, California, and South Dakota.

The long-term goal of the research has been to enhance understanding of the evolution of hormone-mediated phenotypes by taking both an experimental and correlative approach to the evolution of life histories. The experimental approach has been to manipulate hormones, testosterone (T) in particular, and then to measure the effects of the manipulation on phenotype and fitness in male and female juncos.

Our focus was the trade-offs between mating and parental behavior and between

reproduction and survival, and our approach was to compare the behavior, physiology, and performance of manipulated and control phenotypes. This comparison allowed us to quantify how selection maintains adaptive distributions of phenotypes (e.g., if the manipulated phenotypes have lower fitness than controls) or whether organisms are sometimes hormonally constrained in their ability to respond to selection (if altered phenotypes have higher fitness than controls). The latter possibility might hold, at least in ecological time, because of the tendency of hormones to produce pleiotropic effects, some of which may be disadvantageous if hormones are altered. For example, if males would benefit from higher levels of testosterone, but the consequences for females would be disadvantageous, correlated responses to selection could constrain male evolution.

More recently our approach has been to document individual, sex, and population-level variation in the ability to produce testosterone. We began by assessing variation among males in one population in their tendency to elevate testosterone naturally in response to a standardized upstream hormonal stimulus, a challenge with gonadotropin releasing hormone (GnRH). We then related variability in this capacity to elevate testosterone in response to GnRH to key phenotypic traits, concentrating on traits already known to be affected by experimental elevation of testosterone, and to fitness. We found some very interesting similarities and differences between the phenotypic and fitness consequences of experimentally elevated T and naturally varying ability in the capacity to produce T.

Most recently we have expanded the GnRH challenge approach to females in the first population we studied in Virginia, and to males and females of other junco populations residing in California and South Dakota.

In addition to the GnRH approach, we have begun to compare juncos for a) the hormonal response of females to environmental stimulation (simulated territorial and predator intrusions, b) the response to GnRH of the pituitary as measured by LH in both males and females, and b) the sensitivity to T of target tissues in the brain. The goal is to ?build a map? of sequential links of stimulus and response that gives rise to variation in androgen-mediated traits in the junco. By concentrating effort on environmental triggers, physiological integration, and variability in target sensitivity, we hope to provide a concrete picture of variation in hormone-mediated trait expression that can be compared to both genomic and quantitative genetic understandings of phenotypic variation.

This report summarizes our most recently activities and findings as supported under this award.

Specific objectives
The proposal that led to this award cited four objectives:

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness. Resemblance between males and females in their phenotypic sensitivity to testosterone will be used to predict potential for direct and correlated responses to selection. Traits that respond to elevated testosterone in both sexes but are harmful to one will be interpreted as evidence for constraint. Ongoing analysis of already collected data will compare testosterone-treated females to controls for extra-pair mating and survival.

Objective 2: To assess individual variation in hormonal responsiveness and relate that variation to phenotype and fitness. Use of a standardized challenge to the hypothalmo-pituitary-gonadal (HPG) axis with gonadotropin releasing hormone (GnRH) has revealed significant co-variation between natural testosterone levels and mating/ parental effort. Research will extend to females using yolk testosterone as a measure of hormonal phenotype. Planned studies will relate male response to GnRH to male phenotype and fitness, and yolk T to female phenotype and fitness.

Objective 3: To compare populations for variation in hormonal responsiveness and testosterone-mediated characters. Suites of hormonally correlated characters may

permit rapid adaptation to new environments; tight linkage of traits to a hormone signal may retard response. Research will assess degree of phenotypic integration and independence by measuring testosterone response to GnRH and relating it to phenotype in three new populations.

Objective 4: To assess variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence. Trait evolution involves changes in both hormone signal and target response. Research will use cellular and molecular techniques to compare neural responses to testosterone and its metabolites in strong and weak responders to GnRH.

Research training
Under these objectives, the award provided funds to help support research training for 2 post-doctoral students (Danielle Whittaker, Kim Rosvall), 8 graduate students (Jonathan Atwell, Christy Bergeon, Kristal Cain, Nicki Gerlach, Dawn O?Neal, Mark Peterson, Dustin Reichard, and Rachel Hanauer).

During 09-10, it supported 6 undergraduates during the academic year (Elizabeth Ansert, Ediri Mitiri, Becky Rice, Elizabeth Swanger, and Meelyn Pandit).

During the summer of 2010, it contributed to the training of 4 REU undergraduate students (Natasha Tonge, Tamara Fetters, Carla Vanderbilt, Meelyn Pandit), and 3 summer field assistants in the period between undergraduate and graduate school during the summer of 10 (Sarah Wanamaker, Matt Boser, Rachel Hanauer), and 1 high school student who will begin as an undergraduate at Indiana U in 2010 (Ingrid Feustel).

During 2010-2011 it helped to train 3 academic year undergraduates (Joe Welkin, Sonya Jayaratna, and Erin Johnson), During summer 2011the award helped to support 3 summer REU students: one primarily supported by a site award to Indiana U (Sonya Jayaratna), one primarily by a site award to the U of Virginia (Cari Lynn Squibb, Virginia Tech University), and one by a supplement to this award (Stephen Fergusen, Wooster College). In addition it helped to support and train 2 summer field assistants (Megan Shreve and Marine Drouilly).

Activities in relation to these specific objectives
Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness.

We captured close to 100 females during each of the years 2005, 2006, and 2007 and treated them with subcutaneous implants of T or with empty implants to produce controls. We released them and observed their behavior at the nest (e.g., parental behavior, nest defense) and measured fitness. The implanting phase of this work is over, and our major activity during 2009-10 was to analyze data on relative fitness of the two classes of females, particularly with respect to extra-pair mating. The primary person

engaged in this research was Nicki Gerlach and she completed her thesis and will spend the coming year publishing these data.

During 2010-11, Dr. Gerlach completed the genotyping of offspring produced by T and C-females and produced the first draft of a manuscript describing the fitness consquences of experimentally elevate testosterone. She also gave an oral presentation at the joint meeting of the International Ethological Congress and the Animal Behavior Society in July

2011.

Objective 2: To assess individual variation in hormonal responsiveness and relate that variation to phenotype and fitness.

The objective here has been to quantify variation among individuals in their capacity to elevate testosterone when challenged with a releasing hormone, gonadotropin releasing hormone (GnRH) and to see whether high or low capacity relates to fitness. GnRH ordinarily stimulates the release of luteinizing hormone (LH), which leads to the release of testosterone (T). We captured individuals in the field in Virginia, bled them to determine pre-challenge levels of T, injected (challenged) them with GnRH, collected blood samples 30 min post-challenge, and measured T. We then related the rise in T to attributes of the birds, including parental behavior, aggression, and plumage coloration and immune function.

The critical next step has been to see whether strong or weak responders differ from one another in survival and/or reproduction. Our chief activity in 09-10 was to complete a manuscript which was published in the American Naturalist and which reports that selection on T in response to GnRH is stabilizing with respect to survival and slightly but non-significantly directional with respect to reproduction. We have compared these results to those arising out of many years of relating phenotypes induced via experimental elevation of T to fitness. The primary person engaged in this research was former graduate student Joel McGlothlin.

We also documented variation in female juncos in T in response to GnRH and related that variation to aggression at the nest and morphology to determine whether androgynous females have higher or lower fitness as measured by survival and reproductive success. We have found that strong responders to GnRH are also more aggressive towards intruders at the nest. The primary person engaged in this research is Kristal Cain.

During 200-11, Kristal Cain had 3 manuscript published and gave presentation at the International Society for Behavioral Ecology in Australia and the IEC-ABS in Bloomington IN

Objective 3: To compare populations for variation in hormonal responsiveness and testosterone-mediated characters.

During 09-10, we continued to study two populations of juncos derived from southern California, one from the campus of the University of California, San Diego, and one from the nearby Laguna Mountains, both of which were held in a ?common garden? in Bloomington, IN. These populations are of particular interest because they are known to have diverged from one another during the past 25 years after the UCSD campus was colonized in the mid-1980s by juncos believed to have derived from a region that include Laguna Mountain.

We compared the captive birds for seasonal variation in T in response to GnRH, corticosterone in response to handling stress, timing of molt, fattening and Zugunruhe, and behavior, both aggression and boldness. We also compared them for migratory restlessness and fattening. The primary person engaged in this research is graduate student Jonathan Atwell.

We learned that the UCSD birds produce lower peak levels of T but are exposed to T longer because they have a longer breeding season. They are less aggressive, more bold, more parental. They are also less likely to enhance fecundity via extra-pair offspring. In addition, they respond less strongly to stressors, have more robust immune responses and are less susceptible to disease. They also show less nocturnal restlessness during migration and fatten less as well. Thus the colonizing population shows a highly coordinated response to a changed environment, much of which could have been predicted

based on their weaker response to GnRH. It is the ability of a change in one hormonal parameter signal to predict so much about the life history of a newly colonizing population that makes this work so extraordinary. Atwell has prepared a manuscript summarizing his data from the field and the common garden that will be submitted to Nature in August 2010.

A powerful next step would be to compare juncos that occupy a more challenging environment in terms of novelty or severity of climate to determine whether we can predict the pattern of hormone secretion and associated phenotypic characters prior to study, based on results arising out of comparisons made to date.

During 2010 we also summarized data on T in response to GnRH and aggressive behavior in population of juncos that resides in the Black Hills of South Dakota. We learned that the Black Hills population elevates T more strongly in response to GnRH and is more aggressive towards an intruder as would have been predicted. We did not find within-population variation between T and behavior as we have found in other populations, a result we are still assimilating. The primary person engaged in this research is graduate student Christy Bergeon.

This work continued during 2010-11. Christy Bergeon completed a paired comparison of Virginia and South Dakota juncos to determine how they vary under common circumstances in T in response to GnRH, LH in response to GnRH, and m-RNA expression of aromatase and androgen receptor in key brain regions. These data are currently being analyzed for Christy?s Ph.D. thesis.

During 2010-11, the California project also moved forward as Jonathan Atwell completed his Ph.D thesis and the summary comparing the two populations in the field and in a common garden. He submitted his findings to Nature, Science, and PNAS, because we thought they merited a venue of that order, but the journals said no, so Atwell is in revising the manuscript.

Objective 4: To assess variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence.

We made very strong progress towards this objective during 2010. First, two people associated with the award, Chirsty Bergeon Burns and Kim Rosvall, visited the laboratory of Barney Schlinger at UCLA to learn micro-dissection and quantitative PCR. Dr. Rosvall then collected male and female juncos in Virginia after assessing their behavioral response to an intruder, and she used the qPCR method to quantify mRNA for androgen receptor (AR), estrogen receptor (ER) and aromatase (AROM) in 2 brain regions, hypothalamus and medial amygdala. She found significant correlated individual variation in brain and behavior in the field (!). This same brain tissue will also be surveyed for receptor density using immunocytochemistry in the coming year.

Christy Bergeon Burns conducted a study to determine which links in the hypothalamic-pituitary-gonadal (H-P-G) axis account for individual variation in the production of testosterone, i.e., do individuals vary more in the degree to which they elevate LH in response to GnRH or T in response to LH? Burns brought juncos from South Dakota to Bloomington, held them over the winter, and measured their response to GnRH in terms of both LH and T. She found individual variation at each level that was correlated with the production of T. She will next compare the gonadal tissues for AR, ER and aromatase. She is also in the process of collecting a series of individuals from Virginia, which will allow a geographic comparison in the coming year.

During 2010-2011, the progress was also rapid. Dr. Rosvall and Christy Burns perfected the qPCR to allow them to quantify AROM, AR and, most recently with new primers, ER. They trained students to help and burned through many samples. They have shown, for

example, co-variation in the intensity of aggressive behavior and the quantity of AROM and AR in the hypothalamus and the nucleus teniae. They also compared the sexes for response to a territorial intrusion and found striking similarities.

During he field season of 2011 Dr. Rosvall compared the impact of brief and prolonged behavioral challenges on the transcriptional responses of key brain regions of birds observed and captured in the field.

Dr. Rosvall was a symposium speaker at the annual meeting of the Society for Behavioral Neuroendocrionology and presented her newest data, and Ms Burns gave a presentation at the joint IEC-ABS meeting in July on her latest data.

As part of objective 4 and with additional funding we have begun to compare gene expression in male and female juncos treated with testosterone.

Additional research and educational activities not stated in the award proposal that have been facilitated because of the existence of NSF funding.

1) Seasonal, sexual, and population- and species-level variation in volatile compounds found in preen oil. Using HPLC, we compared the volatile composition of preen oil in juncos by sex, season and population and in an array of species breeding in southern Indiana. We also measured the impact of preen oil on incubation behavior and laid the groundwork to relate variation in preen oil composition to variation in the major histocompatibility complex (MHC). We are collaborating with Milos Novotny and Helena Soini. The primary person engaged in this research is post-doctoral student Danielle Whittaker and she published 2 papers during 2009 and 2010 and two more papers in 2011

2) Junco transcriptome. We have produced a compiled and partially annotated transcriptome for the junco. We are collaborating with John Colburne of the IU Center for Genomics and Bioinformatics. The primary person engaged in this research is graduate student Mark Peterson, and the annotation was completed in 2011. The results are in manuscript form and will soon be submitted to GMC genomics. The transcriptome was used to generate a gene array and we are using it to compare impacts of testosterone on gene expression.

3) Documentary film of junco research. We have begun to produce an engaging educational multimedia project that will serve to enhance public understanding of evolution and also serve to document our past and current research on the junco. The junco is a classic species for studies of speciation and environmental physiology and is also the subject of our ongoing research on the evolution of complex (hormone- mediated) phenotypes, the expression of aggressive and parental behavior, sex differences in migratory behavior, and the responses of bird populations to climate change and urbanization. During 2010 we collected footage in California, Guatemala, and Mexico, including Guadalupe Island. The primary person engaged in this project is Jonathan Atwell and videographer Steve Burns, along with graduate student Christy Bergeon Burns.

During 2011 the film crew went to Baja Sur at the southern tip of Baja to film an endemic junco and made filming expeditions to San Diego and to Teton National Park in Wyoming. The footage has been documented and the real work has begun. This project is now funded by an OPUS proposal but began under this award.

4) Vocal behavior in the dark-eyed junco and its potential role in population divergence. Juncos produce two types of song. One is the loud territorial song that it is referred to as long range song (LRS). The other type is produced at low volume, is complex, is sung during courtship, and is referred to as short-range song (SRS). The territorial song is quite similar geographically, and males in the eastern US respond equally

strongly to playbacks of eastern and western LRS (unpublished data). Much less is known about the ?meaning? of SRS, whether it varies geographically, and how it impacts female reproductive physiology. We are comparing the structure and function of LRS and SRS within and among populations of juncos and during 2010 we began to collect recordings from a new population of juncos, the pink-sided junco in Wyoming.

We are also using miniature transmitting microphones to track the vocal behavior of free-ranging juncos in collaboration with Dr. Dave Enstrom of the U of Illinois.

During 2011 we wrote up the first paper summarizing differential responses of male juncos to playback of long and short range song, a paper that will appear in American Naturalist.

The primary person engaged in this project is graduate student Dustin Reichard.

5) Migratory behavior in the dark-eyed junco. Juncos differ in the distance they migrate in autumn by sex, and females migrate farther than males. We have asked whether recent climate warming has had an influence of migration distance and our data show that female juncos have apparently shortened their migrations, perhaps in response to climate warming. We had anticipated that immune function might vary geographically or as a function of competition between the sexes but did not find support for this hypothesis. The primary person engaged in this project was graduate student, Dawn O?Neal, who completed her Ph.D on this project in 2010.

In 2010-2011 we participated in a workshop that attracted legal scholars, conservation biologists and migration biologists. We discussed how best to preserve migrations and published the results of our findings in an environmental law journal.

Jonathan Atwell was the lead on this project.

Findings: (See PDF version submitted by PI at the end of the report)

MAJOR FINDINGS OF THE RESEARCH 2011.These findings are presented in relation to the objectives described in the proposal, using the same order as the activities file and the same headings. Published findings are primarily referred to as citations; as yet unpublished findings are presented as text or graphs; if there were no new findings in 2011, nothing is reported.

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness. Resemblance between males and females in their phenotypic sensitivity to testosterone will be used to predict potential for direct and correlated responses to selection. Traits that respond to elevated testosterone in both sexes but are harmful to one will be interpreted as evidence for constraint. Ongoing analysis of already collected data will compare testosterone-treated females to controls for extra-pair mating and survival.

Dr. Nicole Gerlach, former graduate student and current post-doctoral student has advanced her summary of data on the relative fitness of testosterone-treated and controls females (T- and C-females), a preview of which was included in last year?s annual report. She has found decreased fecundity in females treated with testosterone, but no difference in numbers of mates or annual survival (rate of return to breed). In order to determine

when in the reproductive cycle the suppressive effects of testosterone on fecundity were taking place, she compared T- and C-females according to stage of reproduction.
She found much less impact of T on fecundity later in the reproductive cycle. The two treatment groups had similar reproductive success during later stages of reproduction and the major effect of T occurred during egg laying. This early but not late effect of T on fecundity could be for at least two reasons.
First, in the population of females as a whole, the hormonal mechanisms that underlie behavior later in the reproductive cycle (e.g. feeding or defending young) may be less affected by testosterone than the mechanisms that underlie behaviors that occur earlier (e.g., nest-building or egg laying).
Alternatively, females in the population may differ in their sensitivity to the suppressive effects of testosterone regardless of stage of reproduction, and those that are most sensitive may cease reproduction sooner, while those that are not sensitive (or are less sensitive) may continue to reproduce and not differ in fecundity from controls.
Either interpretation would appear as lesser suppression of reproduction by T in later stages of reproduction, which is what we found. And both outcomes would of interest with respect to the neuroendocrine mechanisms underlying reproductive physiology and behavior, but they have somewhat different evolutionary implications.
The data appear in Figure 1 below and were presented by Dr. Gerlach in a talk at the joint meeting of the International Ethological Conference and the Animal Behavior Society in July 2011. We now have a manuscript draft in hand for early submission. Future work will attempt to distinguish between the two working hypotheses (population as a whole less sensitive to T in later stages of reproduction vs. females that are inherently less sensitive to T reproduce at the same rate as controls) by conducting longitudinal studies on females treated at different stages of reproduction.
Figure 1. Reproductive success of female dark-eyed junco by hormone treatment (C implants = black bars; T implants = white bars). A) All implanted females. B) Females that reached to the previous reproductive stage (e.g. the mean number of eggs among females that built at least one nest, the number hatched among females that laid at least one egg, etc.). C) Females that reached the reproductive stage in question (e.g. the mean number of eggs among females that produced at least one egg, the mean number of hatching among females that produced at least one hatchling).

Objective 2: To assess individual variation in hormonal responsiveness and relate that variation to phenotype and fitness. Use of a standardized challenge to the hypothalmo-pituitary-gonadal (HPG) axis with gonadotropin releasing hormone (GnRH) has revealed significant co-variation between natural testosterone levels and mating/ parental effort. Research will extend to females using yolk testosterone as a measure of hormonal phenotype. Planned studies will relate male response to GnRH to male phenotype and fitness, and yolk T to female phenotype and fitness.

Findings relating to this objective as applied to males were published in 2010 and were described in last year?s report. (McGlothlin, J.W., Whittaker, D.J., Schrock, S.E., Gerlach, N.M., Jawor, J.M., Snajdr, E.A., and E. D. Ketterson. 2010. Natural selection on testosterone production in a wild songbird population, Am Nat 175: 687-701 DOI: 10.1086/652469).

Studies of females are still underway. We have not yet related yolk T to female phenotype and fitness though the eggs have been collected and the assays will be conducted this year.

We have related female aggression to female T in response to GnRH, and we found that females that responded more strongly to GnRH by elevating T were also more aggressive

during staged intrusions by conspecifics.

These findings were presented at the International Symposium for Behavioral Ecology in Australia during autumn 2010 and are will appear in Behavioral Ecology and Sociobiology (BES) in an article by Kristal Cain and Ellen Ketterson, 'Competitive females are successful females; phenotype, mechanism and selection in a common songbird.'

A set of figures from the BES manuscript appears below. The upper two panels plot female morphology (body size) on the x-axis and elevation of T in response to GnRH on the y-axis. The lower two panels related elevation of T to individual variation in female aggression. The findings indicate that bigger and more aggressive females are also ?high T? females.

Cain, K.E. and E.D. Ketterson, 2011. Competitive females are successful females; phenotype, mechanism and selection in a common songbird. Behavioral Ecology and Sociobiology, in press.

Cain, K.E, Rich, M.S., Ainsworth, K., and E. D. Ketterson. 2011. Two sides of the same coin? consistency in aggression to conspecifics and predators in a female songbird. Ethology, 117: 786?795. doi: 10.1111/j.1439-0310.2011.01932.x

Objective 3: To compare populations for variation in hormonal responsiveness and testosterone-mediated characters. Suites of hormonally correlated characters may permit rapid adaptation to new environments; tight linkage of traits to a hormone signal may retard response. Research will assess degree of phenotypic integration and independence by measuring testosterone response to GnRH and relating it to phenotype in three new populations.

Last year we reported the highlights of our findings based on comparisons between two populations in California, one ancestral residing in native habitat in the mountains near San Diego, and a second that recently colonized the city of San Diego and is clearly urban. Prior work by Trevor Price and his students, particularly Pamela Yeh, had demonstrated population differences in timing of reproduction, brood number, aggression, body size and ornamental plumage. They had also shown that the body size and plumage differences persisted in a common garden.

Graduate student Jonathan Atwell working in collaboration with Goncalo Cardoso, a post-doc with Trevor Price, and then post-doctoral student Danielle Whittaker, found that these populations also differed in parental behavior, immune function, corticosterone (baseline and in response to restraint), behavioral boldness, plumage coloration, and frequency of extra-pair fertilizations.

Because most of these traits had previously been shown to be affected by experimental elevation of testosterone in the population we study in Virginia, we predicted that the populations would also differ in testosterone and that testosterone would be higher in the mountain population that is less parental, more aggressive, has brighter plumage, higher corticosterone, and reduced immune function. As predicted, we found in the field that testosterone after a simulated territorial intrusion and after an injection with GnRH was higher in the mountain population than in the colonist population.

Importantly we also observed co-variation between T and phenotype at the level of individuals within each population, e.g., higher T/more aggressive and vice-versa, higher T/less parental and vice versa. Further, the two populations were arranged along the same axes of co-variation, e.g. individuals from the more aggressive and less parental population had higher levels of T, which could be interpreted as moving along phenotypic

lines of least resistance or as rapid adaptive modification based on correlated traits with a common hormonal mediator.

These patterns suggest that a change in mean signal strength (circulating levels of testosterone) between populations could explain at least a part of the observed population differences in behavior and physiology. This was extremely satisfying to learn because it suggested that tight integration of correlated phenotypic traits with a hormone signal could allow for rapid adjustment to changing environments.

These findings leave at least three important questions unanswered. First, should the documented differences in hormone and phenotype be attributed to genetic divergence or to phenotypic plasticity in hormone mediation? Second, are the observed phenotypic differences a direct reflection of variation in T or are there other hormones involved? Third, what is it about the environment in San Diego that apparently favors lower T?

To address the first and second questions, Jonathan Atwell raised birds from both populations in a common garden and found that many of the phenotypic differences were maintained, consistent with what would be expected if differences between populations have a genetic basis. Thus birds in the common garden differed by population in immune function, plumage, boldness, and levels of corticosterone. The boldness difference is shown here.
Somewhat to our initial surprise, however, the hormone testosterone tended to converge in the common garden, suggesting that the T-mediated differences in phenotype may result from plastic phenotypic responses to the different environments in the field.

The population level difference in corticosterone persisted at a near significant level suggesting a) the phenotypic differences between populations may involve corticosterone as well, and b) those differences may be genetic in nature. Data slides are presented here:

As to the third question, what are the environmental drivers of population divergence, whether in phenotype or in genotype, a number of possibilities exist that may be difficult to separate. The San Diego environment is noisy and populated by people. The climate is Mediterranean, leaving more time for breeding and prolonged exposure to elevated testosterone. The disease environment is likely to be more challenging simply because of the milder climate. All of these factors could act on one or more hormone-mediated traits in a directional way leading to correlated responses in other traits. For example, if the disease environment requires greater investment in immune function, a reduction in T that leads to a re-allocation of time and energy toward self-maintenance may be favored, particularly in a climate that allows prolonged reproduction. Alternatively, the non-native resources in the city may require greater parental effort on the part of males to provide for offspring, again given prolonged reproduction. If more parental males were favored via a reduction in testosterone, some of the other trait differences would be expected to follow.

The findings of this series of studies were submitted to Nature and rejected without review and then to Science where the manuscript was reviewed but ultimately rejected.

Atwell, J.W. Cardoso, G. C., Whittaker, D.J. Price, T.D. and E. D. Ketterson 201x. Testosterone mediates successful establishment of a songbird population in a novel environment. Submitted to Science, under revision for another journal.

We are currently revising the manuscript for submission to another journal, probably American Naturalist. In the meantime the lead author, Jonathan Atwell completed his Ph.D.

thesis this summer and these data results appear in the thesis.

A second manuscript is also ready for submission and it will focus on corticosterone, the steroid that did not converge in the common garden. We found in the field that the recently diverged urban population is less responsive to stressors (lower CORT after handling) and that difference is also evident in captives held in a common garden. Further the field birds in San Diego allow a person to approach more closely before taking flight than do the birds in the mountains, and birds in the common garden are more exploratory under controlled conditions if they are from San Diego vs. the the mountain/

It is possible that while expecting evolutionary divergence in testosterone we found plasticity, but unexpectedly were presented with findings that are consistent with evolutionary divergence in corticosterone.

We are considering future studies to compare 1) the disease environments, 2) more sophisticated measures of boldness behavior, and 3) whether our results might generalize to other coastal breeding populations of the junco.

Objective 4: To assess variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence. Trait evolution involves changes in both hormone signal and target response. Research will use cellular and molecular techniques to compare neural responses to testosterone and its metabolites in strong and weak responders to GnRH.
Graduate student Christy Bergeon Burns and post-doctoral student Kim Rosvall have been in charge of this objective. We pursued this goal by quantifying receptor m-RNA for androgen receptor (AR) and the enzyme aromatase (AROM) in two neural tissues, the ventral medial telencephalon (VmT) which contains the medial amygdala (the mammalian homolog to the avian nucleus teniae, nT), a region associated with aggression, and the hypothalamus (HYPO) a region related to the regulation of reproduction among many other functions.
Focusing first on Dr. Rosvall?s work, she presented free-living juncos of both sexes in Virginia with a same-sex intruder and quantified their behavior immediately prior to sacrifice. She then flash froze the brains for later processing, and instruction from Barney Schlinger?s lab at UCLA, imported quantitative PCR techniques to Indiana.
She has demonstrated individual variation in relative m-RNA in VmT and HYPO that correlated with behavior. Males that flew over the intruder more often had higher levels of AROM transcript and AR transcript in the VmT, and males that sang more frequently had lower levels of AROM and AR transcript in the HYPO, as shown below.

We do not yet have ready interpretations of these patterns, greater AR and AROM in the VmT of birds that perform my flyovers is consistent with greater aggression. Why transcripts in HYPO would correlate with singing frequency, particularly negatively, will require more consideration.
Christy Burns and Kim Rosvall have collaborated on a related project, which is comparing behavior, morphology, circulating T, and sensitivity to T in two populations that have been separated for far longer than the populations in California. Males from a population in South Dakota belong to a group of juncos known as White-winged Juncos that are larger in body size, more aggressive, and more strikingly marked than the juncos in Virginia or elsewhere in North America.

They employed the same field and laboratory techniques on these two populations - present a male, quantify behavior, sacrifice immediately, flash freeze the brain, later

micro-dissect particular tissues, isolate RNA, make c-DNA, and quantify the c-DNA using qPCR to allow estimations of relative abundance of transcript. The greater the quantity of m-RNA in the original tissue, the fewer PCR cycles required prior to exponential doubling of the transcript. All the proper controls were employed.
The white-winged juncos have higher circulating levels of plasma T than the juncos in VA but they also differ in m-RNA for AR and AROM, which suggests that population divergence in hormone-mediated traits involve modification of hormone signal and hormone response.
While the South Dakota and Virginia juncos differed in abundance of transcript, the differences are not yet readily interpretable. In the HYPO, Virginia juncos had more m-RNA for AR; in the VmT, South Dakota juncos had more m-RNA for AROM. Research on these comparisons is ongoing.

Dr. Rosvall gave a symposium presentation at the Society for Behavioral Neuroendocrinology in Mexico in June 2011, and Christy Bergeon Burns presented her findings at the joint meeting of the International Society for Behavioral Ecology and the Animal Behavior Society in July 2011.

Other findings from 2010-2011 that received some support from this award and also from previous awards by NSF to this ongoing project and from other funding sources.

Chemical ecology in juncos and MHC

Led by Dr. Danielle Whittaker (now of the Beacon Institute at Michigan State University) and in collaboration with Milos Novotny, we have found that juncos vary by sex and population in the composition of volatile compounds produced in their preen glands. Efforts to relate this to MHC were of some but not complete success. We recently learned that junco preen glands produce abundant m-RNA for androgen receptor, so there is much room for future research, some of which will be conducted independently by Dr. Whittaker and some by our group.

Whittaker, D.J. Richmond, K.M., Miller, A.K. Kiley, R. Bergeon Burns, C. Atwell, J.W. and E.D. Ketterson. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (Junco hyemalis). Behavioral Ecology doi: 10.1093/beheco/arr122
Whittaker, D.J., Dapper, A.L., Peterson, M.P., Atwell, J.W. and E.D. Ketterson. 201x. A test of methods for measuring population diversity in passerine MHC Class II. Journal of Avian Biology, under revision.
Whittaker, D.J., Soini, H.A. Gerlach, N.M. Posto, A.L. Novotny, M.V. and E.D. Ketterson. 201x. Limited role of testosterone in stimulating seasonal changes in songbird chemosignal production. Journal of Chemical Ecology, submitted, under revision

Vocal behavior in dark-eyed juncos

Graduate student Dustin Reichard has been investigating the information content in junco song types and recently had a paper appear in American Naturalist. He is also taking his work in a comparative direction, and has added pink-sided juncos in Wyoming to his research.

Reichard, D.G., Rice, R.J., Vanderbilt, C.C., and E.D. Ketterson. 2011. Deciphering information encoded in birdsong: male songbirds with fertile mates respond most strongly to complex, low-amplitude songs used in courtship. American Naturalist 178: 000-000, published on line DOI: 10.1086/661901

In relation to our overall objective of probing the role of testosterone in phenotypic integration and independence, we know that elevation of testosterone causes males to sing

long-range song (full volume) more often. We don?t know whether T affects on short-range song, but it will be interesting to explore because we now know that this type of song can elicit extremely strong aggressive responses from males.

Results of long-term data accumulated with NSF support

One of our long-term goals has been to assess the causes and consequences of extra-pair mating in female birds and whether sexual selection acts on females. These two papers, one submitted and one accepted reflect progress on this goal. Female juncos that produce extra-pair offspring leave more grandchildren than do females that produce only within-pair offspring, which is first report showing differences in fecundity of such offspring as adults. Female juncos that employ more sires in their broods also leave more offspring, giving rise to positive Bateman?s gradients.

Gerlach, N. M., McGlothlin, J.W., Parker, P.G., and E.D. Ketterson. 2011. Promiscuous mating produces offspring with higher lifetime fitness. Proceedings Royal Society B, published on line DOI: 10.1098/rspb.2011.1547

Gerlach, N.M., McGlothlin, J.W., Parker, P.G., and E.D. Ketterson. 201x. Interpreting positive Bateman gradients in female and male dark-eyed juncos. Behavioral Ecology, submitted.

Migratory behavior in the dark-eyed junco

In assessing the role of hormones, particularly testosterone, in population divergence, we have become increasingly aware of the importance of seasonality and physiological adjustments to the timing of breeding and migrating. Jonathan Atwell spearheaded this summary of the diversity of migratory patterns in the junco that was published as part of the proceedings of a workshop on the conservation of migration as a phenomenon of nature.

Atwell, J.W., O?Neal, D.M. and E. D. Ketterson. 2011. Migration as a moving target for conservation: intra-species variation and responses to environmental change, as illustrated in a sometimes migratory songbird, Environmental Law 41(2):289-316.

Training and Development:

Please see sections on activities and findings for graduate and undergraduate students who received research training under this award.

The list of students trained during 2008-09 and 2009-10 and 2010-11 include

Post-doctoral students
Danielle Whittaker 2006-2010
Kim Rosvall 2009-
Nicole Gerlach 2010-
Jonathan Atwell 2011-
Eric Liebgold 2011-

Graduate students
Nicki Gerlach 2003-2010
Jonathan Atwell 2004-2011
Dawn O?Neal 2004-2010
Christy Bergeon Burns 2006-
(co-advised Greg Demas)
Kristal Cain 2006-
Dustin Reichard 2007-

Mark Peterson 2008-
Rachel Hanauer 2010-

Undergraduates
Becky Rice 2008- Cox Scholar, IFLE, STARS, REU (Atwell, O?Neal, Reichard), Indiana University
Meelyn Pandit 2009 STARS, Cox Scholar (Atwell, Reichard), Indiana University
Matt Boser 2009 REU Eastern Connecticut University
Lauren Chaby 2009 REU University
Miram Rich 2009 REU Swarthmore University
Alison Miller 2009 REU University of California San Diego
Natasha Tonge 2010 REU Swarthmore College
Carla Vanderbilt 2010 REU Stetson University
Tamara Fetters 2010 REU Virginia Tech University
Meeylyn Pandit 2010 REU Indiana University
Sonya Jayaratna 2011 undergrad research student and REU Indiana University
Kaitlin Richmond 2011 undergrad research assistant Hanover College
Megan Shave 2011 post-undergrad field assistant

Outreach Activities:

We are creating a media project to promote evolution, animal behavior, and the junco as a model system. We have interviewed biologists in Mexico and the US and obtained footage from Mexico, Guatemala, and various field sites in the US (CA, SD, VA, IN). A link to a trailer promoting the project is here
http://vimeo.com/8871572

McGlothlin et al. 2010 was picked up by the science news
http://www.sciencedaily.com/releases/2010/05/100504102126.htm

Whittaker et al. 2010 was also picked up by the science news
http://www.livescience.com/culture/pheromone-songbird-mating-sl-whittaker-100421.html

During the winter of 2009 students associated with the project made presentations to the local Audubon Society and one of them was picked up by the Bloomington paper, the Herald Times.
Here is the story.
Birds: Not just for amateurs, as IU grad students do avian research to help some species survive
Most birders are hobbyists, ranging from casual to obsessive. Then there are the professionals ornithologists who spend their lives in for ...
2/22/2009

During the spring of 2009 one of the undergraduates in our group was highlighted in a publication entitled the IU Teaching and Learning Magazine.
http://www.indiana.edu/~tandlpub/story.php?story_id=107

In the February/March 2008 issue of National Wlldlife the Backyard Birding section by David Lucas referred to our research
http://www.nwf.org/NationalWildlife/article.cfm?issueID=127&articleID=1694

Our laboratory website provides access to our research, including mounted pdf version of papers
http://www.indiana.edu/~kettlab/

Journal Publications

Atwell, JW; Whittaker, DJ; Ketterson, ED, "Testosterone, social behavior, and ornaments in two recently diverged dark-eyed junco populations", INTEGRATIVE AND COMPARATIVE BIOLOGY, p. E7, vol. 49, (2009). Published,

Cain, KE; Ainswoth, KL; Ketterson, ED, "Is Testosterone a Mediator for Aggressive Behavior in Female Dark-eyed Juncos?", INTEGRATIVE AND COMPARATIVE BIOLOGY, p. E25, vol. 49, (2009). Published,

Ketterson, ED; Atwell, JW, "Phenotypic Integration and Independence: Hormones, Performance, and Response to Environmental Change", INTEGRATIVE AND COMPARATIVE BIOLOGY, p. E90, vol. 49, (2009). Published,

Burns, CMB; Cain, KE; Ketterson, ED, "Phenotypic integration of testosterone-mediated characters across distinct subspecies of the Dark-eyed Junco", INTEGRATIVE AND COMPARATIVE BIOLOGY, p. E199, vol. 49, (2009). Published,

Cardoso, GC; Atwell, JW; Ketterson, ED; Price, TD, "Song types, song performance, and the use of repertoires in dark-eyed juncos (Junco hyemalis)", BEHAVIORAL ECOLOGY, p. 901, vol. 20, (2009). Published, 10.1093/beheco/arp07

Whittaker, DJ; Reichard, DG; Dapper, AL; Ketterson, ED, "Behavioral responses of nesting female dark-eyed juncos Junco hyemalis to hetero- and conspecific passerine preen oils", JOURNAL OF AVIAN BIOLOGY, p. 579, vol. 40, (2009). Published, 10.1111/j.1600-048X.2009.04813.

O'Neal, DM; Swanger, L; Ketterson, ED, "Latitudinal Variation in Winter Immune Function in a Differential Migrant", INTEGRATIVE AND COMPARATIVE BIOLOGY, p. E125, vol. 49, (2009). Published,

Ketterson, E. D., J. W. Atwell, and J. W. McGlothlin., "Phenotypic integration and independence: Hormones, performance, and response to environmental change.", Integrative and Comparative Biology, p. 365, vol. 49, (2009). Published,

McGlothlin, J.W., Whittaker, D.J., Schrock, S.E., Gerlach, N.M., Jawor, J.M., Snajdr, E.A., and E. D. Ketterson., "Natural selection on testosterone production in a wild songbird population]", American Naturalist, p. 687, vol. 175, (2010). Published, DOI: 10.1086/652469.

Whittaker, D.J., Soini, H.A., Atwell, J.W., Hollars, C., Novotny, N.V., and E. D. Ketterson., "Preen oil as a potential chemosignal source in songbirds: individual, sex, and population differences in the volatile compounds of dark-eyed junco (Junco hyemalis) preen gland secretions.", Behavioral Ecology, p. 608, vol. 21, (2010). Published, doi:10.1093/beheco/arq033

Whittaker, D. J., Reichard, D.G., Dapper, A., and E. D. Ketterson., "Behavioral responses of nesting females to hetero- and conspecific passerine preen oils.", journal of avian biology, p. 579, vol. 40, (2009). Published, DOI 10.1111/j.1600-048X.2009.04813.x

McGlothlin, JW; Whittaker, DJ; Schrock, SE; Gerlach, NM; Jawor, JM; Snajdr, EA; Ketterson, ED, "Natural Selection on Testosterone Production in a Wild Songbird Population", AMERICAN NATURALIST, p. 687, vol. 175, (2010). Published, 10.1086/65246

Whittaker, DJ; Soini, HA; Atwell, JW; Hollars, C; Novotny, MV; Ketterson, ED, "Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations", BEHAVIORAL ECOLOGY, p. 608, vol. 21, (2010). Published, 10.1093/beheco/arq03

Books or Other One-time Publications

Web/Internet Site

URL(s):
http://www.indiana.edu/~kettlab/index.html
Description:
Ketterson lab site

Other Specific Products

Contributions

Contributions within Discipline:

Papers with Ketterson, ED as an author were cited 366 times in 2009.
Papers with Ketterson, ED as an author were cited 368 times in 2010
Papers with Ketterson, ED as an author were cited 279 times in 2011 to date

Contributions to Other Disciplines:

Beyond the world of animal behavior and evolutionary biology, our work has been of interest to anthropologists and neuroendocrinologists

Contributions to Human Resource Development:

During 2009, funds from the award helped to support the research of 2 post-doctoral students, 7 graduate students, 4 undergraduate research students during the summer, 3 post-undergraduate field assistants in the summer and 3 undergraduate research students during the academic year.

During 2010, funds from this award helped to support the research of 2 post-doctoral students, 8 graduate students, 5 summer undergraduates and 3 undergraduate research students during the academic year.

During 2011 funds from this award helped to support the research of 2 post-doctoral, 7 graduate students, 6 summer undergraduates, and 5 undergraduate students during the academic year

Contributions to Resources for Research and Education:

During 2009 we were archiving field data, tissue and plasma samples, photos and records from 20 years of this project with the intention of making them available to others in readily accessible format. This will take some time but is a clear objective. We continued this project during 2010, making significant progress in creating an archive of images, data, and electronic media. We are also creating a documentary film project that will be of use to a wide array of adult audiences. Progress on the documentary film was significant in 2011 including field shots from Baja Sur, Wyoming and California.

Contributions Beyond Science and Engineering:

not yet

Conference Proceedings

Special Requirements

Special reporting requirements: None
Change in Objectives or Scope: None
Animal, Human Subjects, Biohazards: None

Categories for which nothing is reported:

Any Book
Any Product
Any Conference

NSF IOB-0820055
Title: Hormones and phenotypic integration: comparing sexes, individuals, and populations, $570,000 plus supplements, 8/01/08-7/31/12

Year 3 report, findings, 8/1/10-7/31/11 (findings are for year 3, years 1 and 2 not repeated)

MAJOR FINDINGS OF THE RESEARCH 2011. These findings are presented in relation to the objectives described in the proposal, using the same order as the activities file and the same headings. Published findings are primarily referred to as citations; as yet unpublished findings are presented as text or graphs; if there were no new findings in 2011, nothing is reported.

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness. Resemblance between males and females in their phenotypic sensitivity to testosterone will be used to predict potential for direct and correlated responses to selection. Traits that respond to elevated testosterone in both sexes but are harmful to one will be interpreted as evidence for constraint. *Ongoing analysis of already collected data will compare testosterone-treated females to controls for extra-pair mating and survival.*

Dr. Nicole Gerlach, former graduate student and current post-doctoral student has advanced her summary of data on the relative fitness of testosterone-treated and controls females (T- and C-females), a preview of which was included in last year's annual report.

She has found decreased fecundity in females treated with testosterone, but no difference in numbers of mates or annual survival (rate of return to breed). In order to determine when in the reproductive cycle the suppressive effects of testosterone on fecundity were taking place, she compared T- and C-females according to stage of reproduction.

She found much less impact of T on fecundity later in the reproductive cycle. The two treatment groups had similar reproductive success during later stages of reproduction and the major effect of T occurred during egg laying. This early but not late effect of T on fecundity could be for at least two reasons.

First, in the population of females as a whole, the hormonal mechanisms that underlie behavior later in the reproductive cycle (e.g. feeding or defending young) may be less affected by testosterone than the mechanisms that underlie behaviors that occur earlier (e.g., nest-building or egg laying).

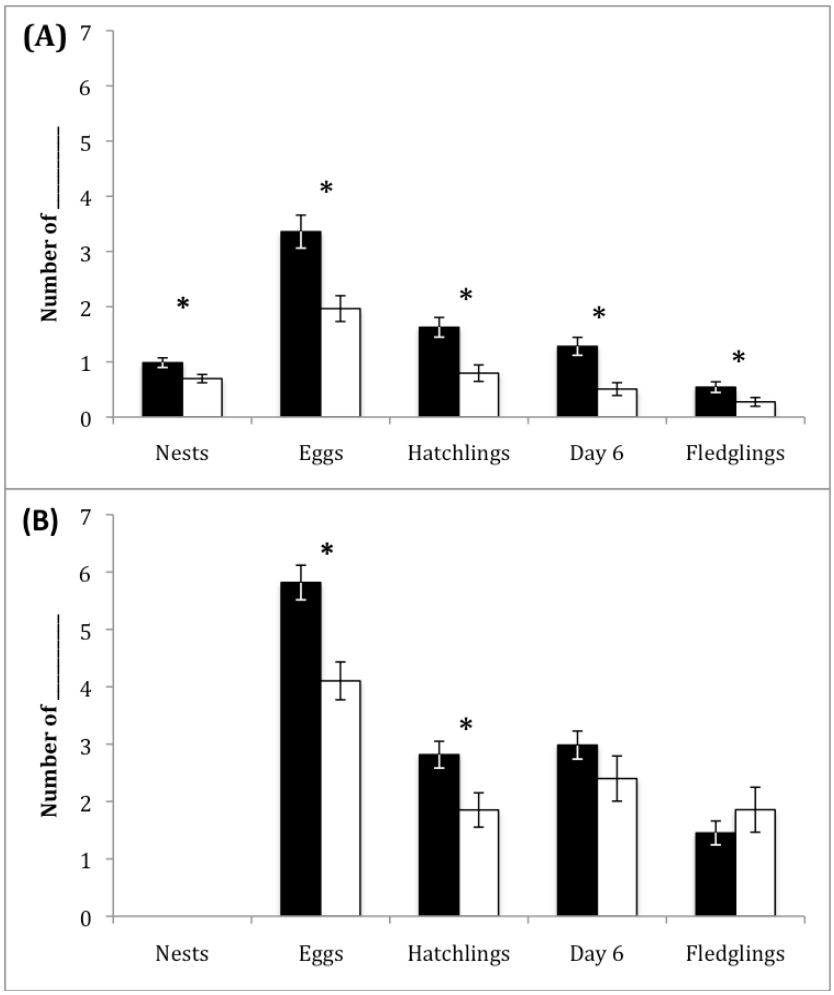
Alternatively, females in the population may differ in their sensitivity to the suppressive effects of testosterone regardless of stage of reproduction, and those that are most sensitive may cease reproduction sooner, while those that are not sensitive (or are less sensitive) may continue to reproduce and not differ in fecundity from controls.

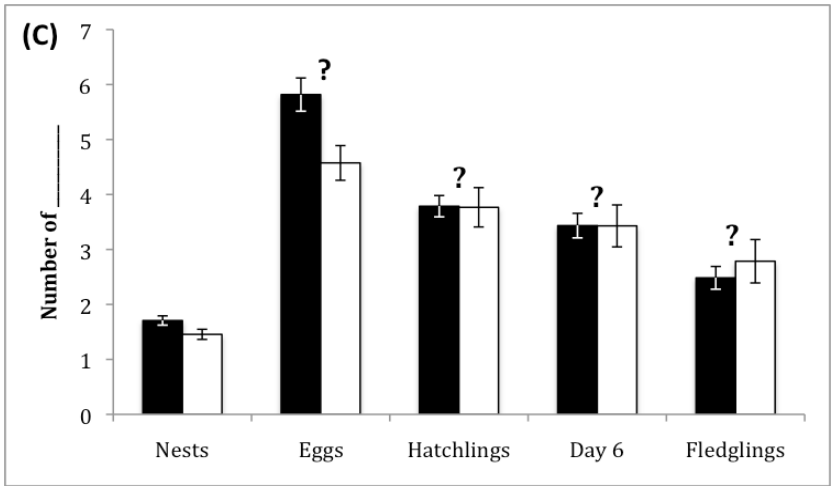
Either interpretation would appear as lesser suppression of reproduction by T in later stages of reproduction, which is what we found. And both outcomes would of interest

with respect to the neuroendocrine mechanisms underlying reproductive physiology and behavior, but they have somewhat different evolutionary implications.

The data appear in Figure 1 below and were presented by Dr. Gerlach in a talk at the joint meeting of the International Ethological Conference and the Animal Behavior Society in July 2011. We now have a manuscript draft in hand for early submission. Future work will attempt to distinguish between the two working hypotheses (population as a whole less sensitive to T in later stages of reproduction vs. females that are inherently less sensitive to T reproduce at the same rate as controls) by conducting longitudinal studies on females treated at different stages of reproduction.

Figure 1. Reproductive success of female dark-eyed junco by hormone treatment (C implants = black bars; T implants = white bars). A) All implanted females. B) Females that reached to the *previous* reproductive stage (e.g. the mean number of eggs among females that built at least one nest, the number hatched among females that laid at least one egg, etc.). C) Females that reached the reproductive stage in question (e.g. the mean number of eggs among females that produced at least one egg, the mean number of hatching among females that produced at least one hatchling).





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Findings relating to this objective as applied to males were published in 2010 and were described in last year's report. (McGlothlin, J.W., Whittaker, D.J., Schrock, S.E., Gerlach, N.M., Jawor, J.M., Snajdr, E.A., and E. D. Ketterson. 2010. Natural selection on testosterone production in a wild songbird population, *Am Nat* 175: 687-701 DOI: 10.1086/652469).

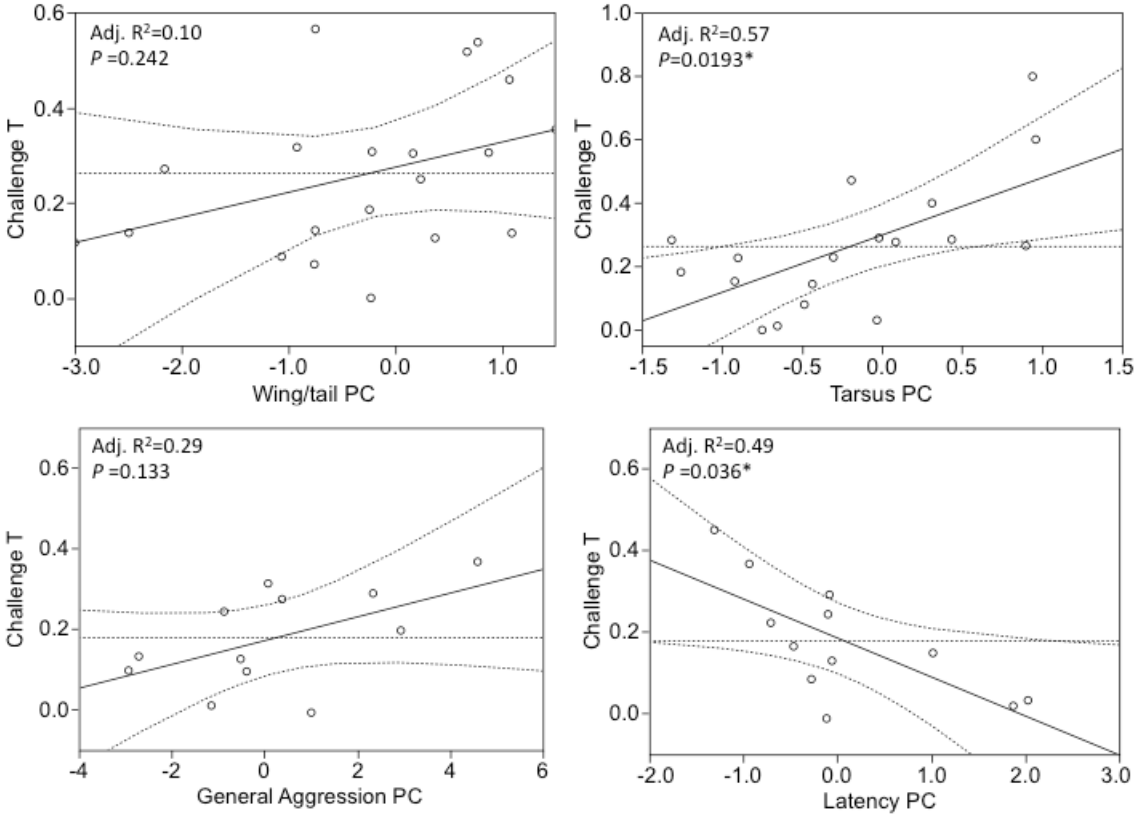
Studies of females are still underway. We have not yet related yolk T to female phenotype and fitness though the eggs have been collected and the assays will be conducted this year.

We have related female aggression to female T in response to GnRH, and we found that females that responded more strongly to GnRH by elevating T were also more aggressive during staged intrusions by conspecifics.

These findings were presented at the International Symposium for Behavioral Ecology in Australia during autumn 2010 and are will appear in Behavioral Ecology and Sociobiology (BES) in an article by Kristal Cain and Ellen Ketterson, "Competitive females are successful females; phenotype, mechanism and selection in a common songbird."

A set of figures from the BES manuscript appears below. The upper two panels plot female morphology (body size) on the x-axis and elevation of T in response to GnRH on

the y-axis. The lower two panels related elevation of T to individual variation in female aggression. The findings indicate that bigger and more aggressive females are also 'high T' females.



Cain, K.E. and E.D. Ketterson, 2011. Competitive females are successful females; phenotype, mechanism and selection in a common songbird. Behavioral Ecology and Sociobiology, in press.

Cain, K.E, Rich, M.S., Ainsworth, K., and E. D. Ketterson. 2011. Two sides of the same coin? consistency in aggression to conspecifics and predators in a female songbird. *Ethology,* 117: 786–795. doi: 10.1111/j.1439-0310.2011.01932.x

Objective 3: To compare populations for variation in hormonal responsiveness and testosterone-mediated characters. Suites of hormonally correlated characters may permit rapid adaptation to new environments; tight linkage of traits to a hormone signal may retard response. *Research will assess degree of phenotypic integration and independence by measuring testosterone response to GnRH and relating it to phenotype in three new populations.*

Last year we reported the highlights of our findings based on comparisons between two populations in California, one ancestral residing in native habitat in the mountains near

San Diego, and a second that recently colonized the city of San Diego and is clearly urban. Prior work by Trevor Price and his students, particularly Pamela Yeh, had demonstrated population differences in timing of reproduction, brood number, aggression, body size and ornamental plumage. They had also shown that the body size and plumage differences persisted in a common garden.

Graduate student Jonathan Atwell working in collaboration with Goncalo Cardoso, a post-doc with Trevor Price, and then post-doctoral student Danielle Whittaker, found that these populations also differed in parental behavior, immune function, corticosterone (baseline and in response to restraint), behavioral boldness, plumage coloration, and frequency of extra-pair fertilizations.

Because most of these traits had previously been shown to be affected by experimental elevation of testosterone in the population we study in Virginia, we predicted that the populations would also differ in testosterone and that testosterone would be higher in the mountain population that is less parental, more aggressive, has brighter plumage, higher corticosterone, and reduced immune function. As predicted, we found in the field that testosterone after a simulated territorial intrusion and after an injection with GnRH was higher in the mountain population than in the colonist population.

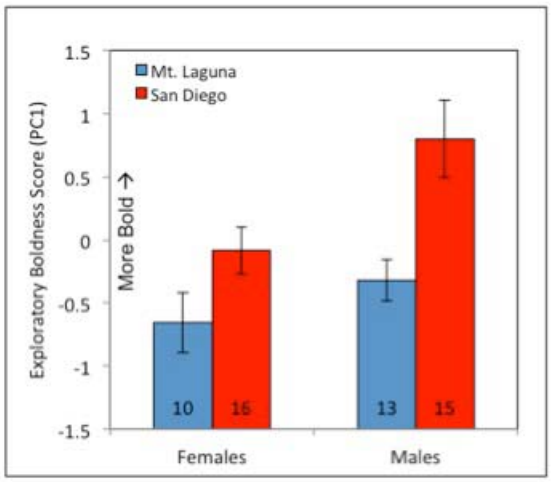
Importantly we also observed co-variation between T and phenotype at the level of individuals *within each population,* e.g., higher T/more aggressive and vice-versa, higher T/less parental and vice versa. Further, the two populations were arranged along the same axes of co-variation, e.g. individuals from the more aggressive and less parental population had higher levels of T, which could be interpreted as moving along phenotypic lines of least resistance or as rapid adaptive modification based on correlated traits with a common hormonal mediator.

These patterns suggest that a change in mean signal strength (circulating levels of testosterone) between populations could explain at least a part of the observed population differences in behavior and physiology. This was extremely satisfying to learn because it suggested that tight integration of correlated phenotypic traits with a hormone signal could allow for rapid adjustment to changing environments.

These findings leave at least three important questions unanswered. First, should the documented differences in hormone and phenotype be attributed to genetic divergence or to phenotypic plasticity in hormone mediation? Second, are the observed phenotypic differences a direct reflection of variation in T or are there other hormones involved? Third, what is it about the environment in San Diego that apparently favors lower T?

To address the first and second questions, Jonathan Atwell raised birds from both populations in a common garden and found that many of the phenotypic differences were maintained, consistent with what would be expected if differences between populations have a genetic basis. Thus birds in the common garden differed by population in immune function, plumage, boldness, and levels of corticosterone. The boldness difference is shown here.

Do population differences in boldness behavior persist in the common garden study?



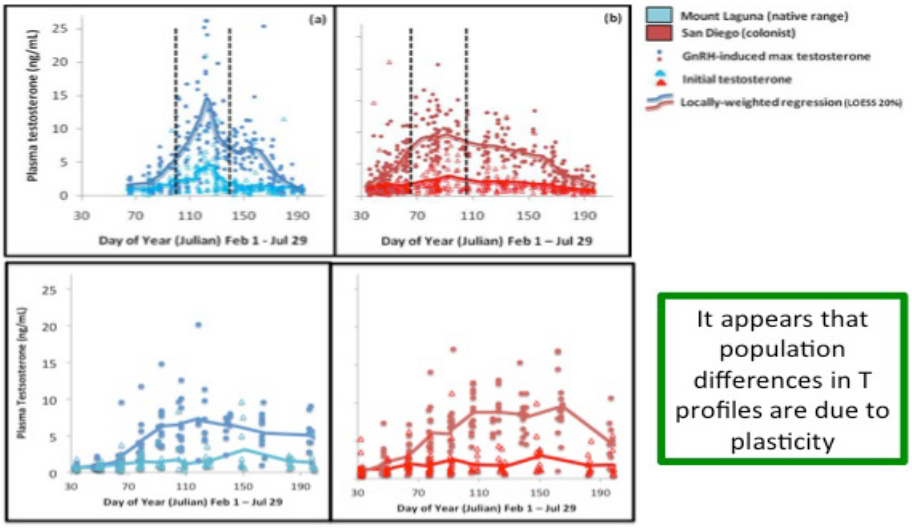
GLM:
Population: F=13.26, p= 0.001
Sex: F=11.43, p=0.044

Somewhat to our initial surprise, however, the hormone testosterone tended to converge in the common garden, suggesting that the T-mediated differences in phenotype may result from plastic phenotypic responses to the different environments in the field.

Do differences in testosterone have a genetic (or early developmental) basis?

Field Study

Common Garden



It appears that population differences in T profiles are due to plasticity

The population level difference in corticosterone persisted at a near significant level suggesting a) the phenotypic differences between populations may involve corticosterone as well, and b) those differences may be genetic in nature. Data slides are presented here:

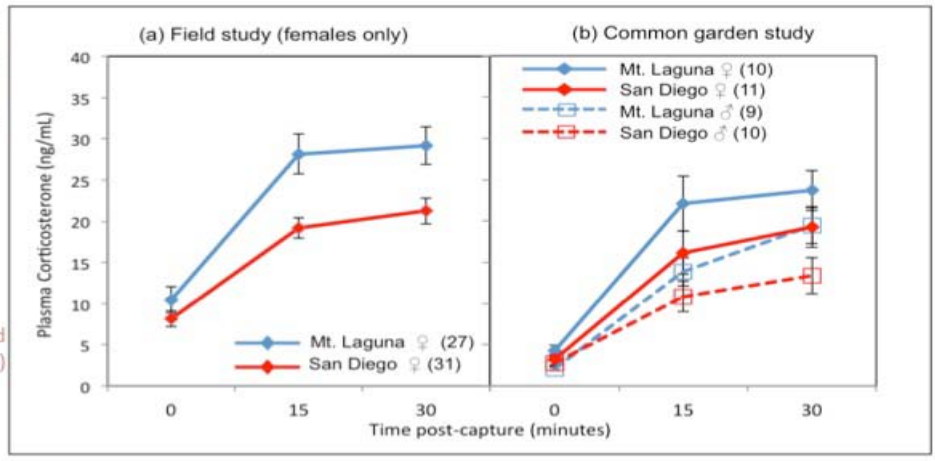
Do population differences in CORT persist in the common garden?

Methods:

Grab focal bird from aviary

Stress-series bleed (0, 15, 30 minutes)

RIA assay



Common Garden GLM:
Baseline CORT: Population, F=0.3, p=0.59; Sex, F=4.42, p=0.043
Cort Response: **Population, F=4.10, p=0.052**; Sex, F=1.99, p=0.169
Max CORT: **Population, F=3.10, p=0.089**; Sex, F=6.33, p=0.017

As to the third question, what are the environmental drivers of population divergence, whether in phenotype or in genotype, a number of possibilities exist that may be difficult to separate. The San Diego environment is noisy and populated by people. The climate is Mediterranean, leaving more time for breeding and prolonged exposure to elevated testosterone. The disease environment is likely to be more challenging simply because of the milder climate. All of these factors could act on one or more hormone-mediated traits in a directional way leading to correlated responses in other traits. For example, if the disease environment requires greater investment in immune function, a reduction in T that leads to a re-allocation of time and energy toward self-maintenance may be favored, particularly in a climate that allows prolonged reproduction. Alternatively, the non-native resources in the city may require greater parental effort on the part of males to provide for offspring, again given prolonged reproduction. If more parental males were favored via a reduction in testosterone, some of the other trait differences would be expected to follow.

The findings of this series of studies were submitted to *Nature* and rejected without review and then to *Science* where the manuscript was reviewed but ultimately rejected.

> Atwell, J.W. Cardoso, G. C., Whittaker, D.J. Price, T.D. and E. D. Ketterson 201x. Testosterone mediates successful establishment of a songbird population in a novel environment. Submitted to *Science,* under revision for another journal.

We are currently revising the manuscript for submission to another journal, probably *American Naturalist.* In the meantime the lead author, Jonathan Atwell completed his Ph.D. thesis this summer and these data results appear in the thesis.

A second manuscript is also ready for submission and it will focus on corticosterone, the steroid that did not converge in the common garden. We found in the field that the recently diverged urban population is less responsive to stressors (lower CORT after handling) and that difference is also evident in captives held in a common garden. Further the field birds in San Diego allow a person to approach more closely before taking flight than do the birds in the mountains, and birds in the common garden are more exploratory under controlled conditions if they are from San Diego vs. the the mountain/

It is possible that while expecting evolutionary divergence in testosterone we found plasticity, but unexpectedly were presented with findings that are consistent with evolutionary divergence in corticosterone.

We are considering future studies to compare 1) the disease environments, 2) more sophisticated measures of boldness behavior, and 3) whether our results might generalize to other coastal breeding populations of the junco.

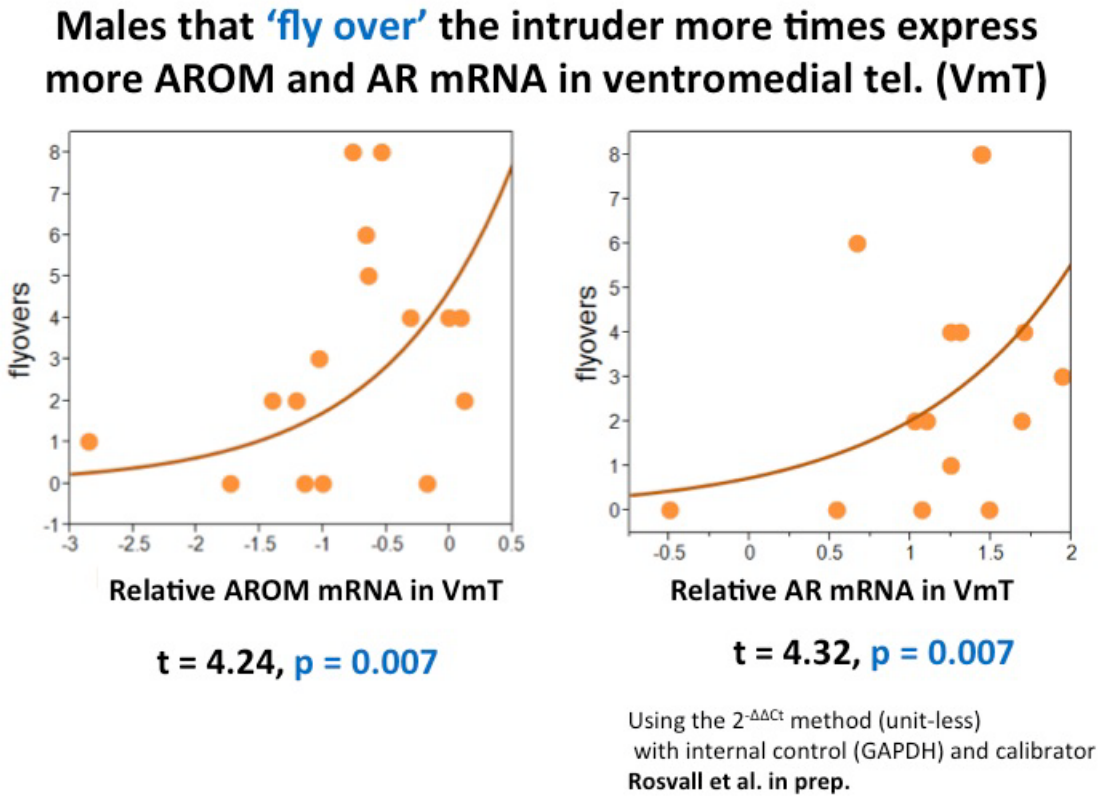
Objective 4: To assess variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence. Trait evolution involves changes in both hormone signal and target response. *Research will use cellular and molecular*

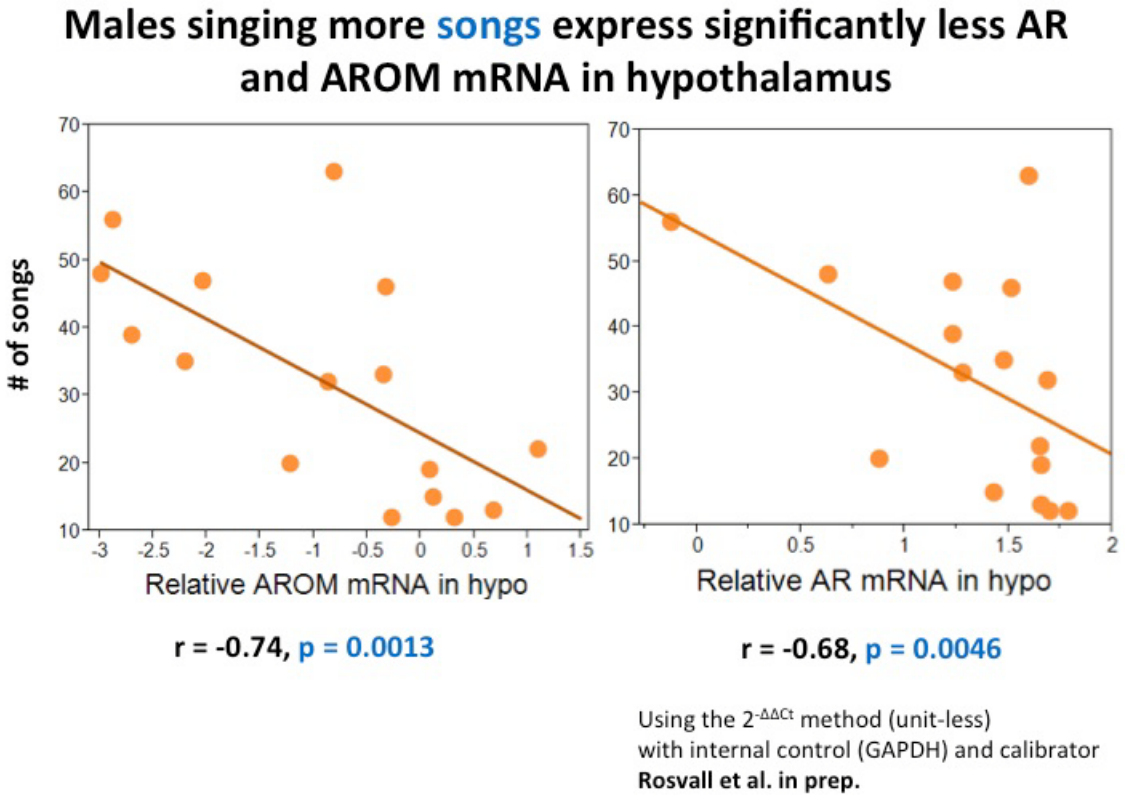
techniques to compare neural responses to testosterone and its metabolites in strong and weak responders to GnRH.

Graduate student Christy Bergeon Burns and post-doctoral student Kim Rosvall have been in charge of this objective. We pursued this goal by quantifying receptor m-RNA for androgen receptor (AR) and the enzyme aromatase (AROM) in two neural tissues, the ventral medial telencephalon (VmT) which contains the medial amygdala (the mammalian homolog to the avian nucleus teniae, nT), a region associated with aggression, and the hypothalamus (HYPO) a region related to the regulation of reproduction among many other functions.

Focusing first on Dr. Rosvall's work, she presented free-living juncos of both sexes in Virginia with a same-sex intruder and quantified their behavior immediately prior to sacrifice. She then flash froze the brains for later processing, and instruction from Barney Schlinger's lab at UCLA, imported quantitative PCR techniques to Indiana.

She has demonstrated ***individual variation in relative m-RNA in VmT and HYPO that correlated with behavior.*** Males that flew over the intruder more often had higher levels of AROM transcript and AR transcript in the VmT, and males that sang more frequently had lower levels of AROM and AR transcript in the HYPO, as shown below.





We do not yet have ready interpretations of these patterns, greater AR and AROM in the VmT of birds that perform my flyovers is consistent with greater aggression. Why transcripts in HYPO would correlate with singing frequency, particularly negatively, will require more consideration.

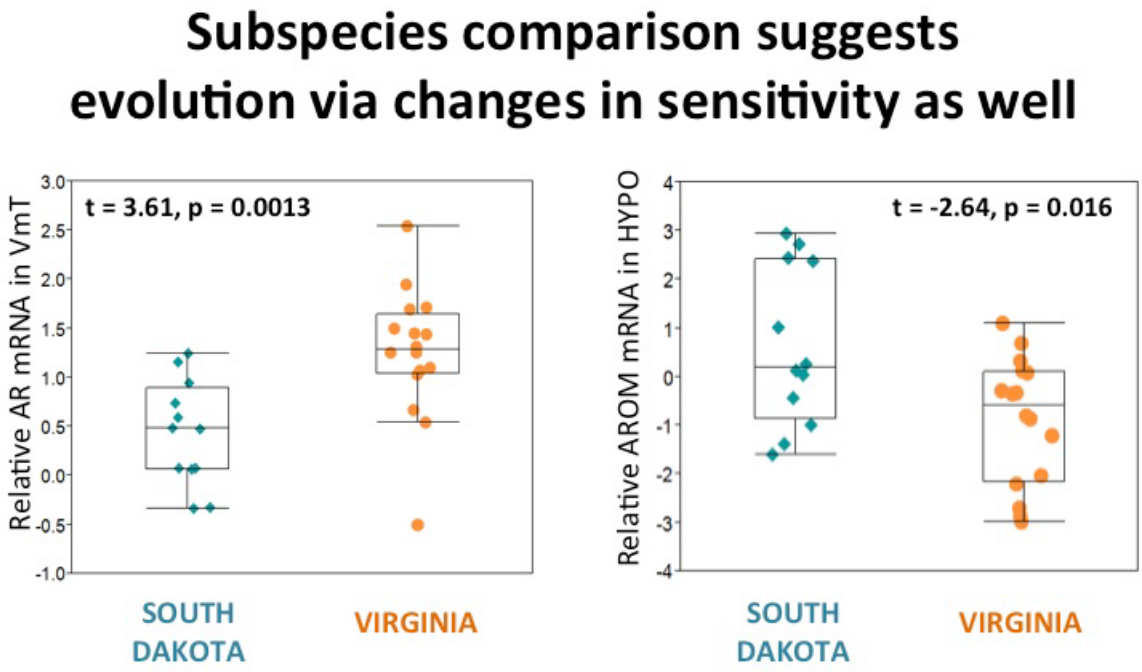
Christy Burns and Kim Rosvall have collaborated on a related project, which is comparing behavior, morphology, circulating T, and sensitivity to T in two populations that have been separated for far longer than the populations in California. Males from a population in South Dakota belong to a group of juncos known as White-winged Juncos that are larger in body size, more aggressive, and more strikingly marked than the juncos in Virginia or elsewhere in North America.

They employed the same field and laboratory techniques on these two populations - present a male, quantify behavior, sacrifice immediately, flash freeze the brain, later micro-dissect particular tissues, isolate RNA, make c-DNA, and quantify the c-DNA using qPCR to allow estimations of relative abundance of transcript. The greater the quantity of m-RNA in the original tissue, the fewer PCR cycles required prior to exponential doubling of the transcript. All the proper controls were employed.

The white-winged juncos have higher circulating levels of plasma T than the juncos in VA but they also differ in m-RNA for AR and AROM, which suggests that population

divergence in hormone-mediated traits involve modification of hormone signal and hormone response.

While the South Dakota and Virginia juncos differed in abundance of transcript, the differences are not yet readily interpretable. In the HYPO, Virginia juncos had more m-RNA for AR; in the VmT, South Dakota juncos had more m-RNA for AROM. Research on these comparisons is ongoing.



VA has more AR in VmT than SoDa, suggesting VA males can "do more with less" T. SoDa birds, which have higher T, may be converting more T to E_2

Bergeon Burns, Rosvall and Ketterson, in prep

Dr. Rosvall gave a symposium presentation at the Society for Behavioral Neuroendocrinology in Mexico in June 2011, and Christy Bergeon Burns presented her findings at the joint meeting of the International Society for Behavioral Ecology and the Animal Behavior Society in July 2011.

Other findings from 2010-2011 that received some support from this award and also from previous awards by NSF to this ongoing project and from other funding sources.

Chemical ecology in juncos and MHC

Led by Dr. Danielle Whittaker (now of the Beacon Institute at Michigan State University) and in collaboration with Milos Novotny, we have found that juncos vary by sex and population in the composition of volatile compounds produced in their preen glands. Efforts to relate this to MHC were of some but not complete success. We recently learned that junco preen glands produce abundant m-RNA for androgen receptor, so there is much room for future research, some of which will be conducted independently by Dr. Whittaker and some by our group.

Whittaker, D.J. Richmond, K.M., Miller, A.K. Kiley, R. Bergeon Burns, C. Atwell, J.W. and E.D. Ketterson. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (Junco hyemalis). *Behavioral Ecology* doi: 10.1093/beheco/arr122

Whittaker, D.J., Dapper, A.L., Peterson, M.P., Atwell, J.W. and E.D. Ketterson. 201x. A test of methods for measuring population diversity in passerine MHC Class II. *Journal of Avian Biology*, under revision.

Whittaker, D.J., Soini, H.A. Gerlach, N.M. Posto, A.L. Novotny, M.V. and E.D. Ketterson. 201x. Limited role of testosterone in stimulating seasonal changes in songbird chemosignal production. *Journal of Chemical Ecology*, submitted, under revision

Vocal behavior in dark-eyed juncos

Graduate student Dustin Reichard has been investigating the information content in junco song types and recently had a paper appear in American Naturalist. He is also taking his work in a comparative direction, and has added pink-sided juncos in Wyoming to his research.

Reichard, D.G., Rice, R.J., Vanderbilt, C.C., and E.D. Ketterson. 2011. Deciphering information encoded in birdsong: male songbirds with fertile mates respond most strongly to complex, low-amplitude songs used in courtship. *American Naturalist* 178: 000-000, published on line DOI: 10.1086/661901

In relation to our overall objective of probing the role of testosterone in phenotypic integration and independence, we know that elevation of testosterone causes males to sing long-range song (full volume) more often. We don't know whether T affects on short-range song, but it will be interesting to explore because we now know that this type of song can elicit extremely strong aggressive responses from males.

Results of long-term data accumulated with NSF support

One of our long-term goals has been to assess the causes and consequences of extra-pair mating in female birds and whether sexual selection acts on females. These two papers, one submitted and one accepted reflect progress on this goal. Female juncos that produce extra-pair offspring leave more grandchildren than do females that

produce only within-pair offspring, which is first report showing differences in fecundity of such offspring as adults. Female juncos that employ more sires in their broods also leave more offspring, giving rise to positive Bateman's gradients.

Gerlach, N. M., McGlothlin, J.W., Parker, P.G., and E.D. Ketterson. 2011. Promiscuous mating produces offspring with higher lifetime fitness. *Proceedings Royal Society B*, published on line DOI: 10.1098/rspb.2011.1547

Gerlach, N.M., McGlothlin, J.W., Parker, P.G., and E.D. Ketterson. 201x. Interpreting positive Bateman gradients in female and male dark-eyed juncos. Behavioral Ecology, submitted.

Migratory behavior in the dark-eyed junco

In assessing the role of hormones, particularly testosterone, in population divergence, we have become increasingly aware of the importance of seasonality and physiological adjustments to the timing of breeding and migrating. Jonathan Atwell spearheaded this summary of the diversity of migratory patterns in the junco that was published as part of the proceedings of a workshop on the conservation of migration as a phenomenon of nature.

Atwell, J.W., O'Neal, D.M. and E. D. Ketterson. 2011. Migration as a moving target for conservation: intra-species variation and responses to environmental change, as illustrated in a sometimes migratory songbird, *Environmental Law 41(2):*289-316.

NSF IOB-0820055
Title: Hormones and phenotypic integration: comparing sexes, individuals, and populations, $570,000, 8/01/08-7/31/12
Year 3 report, research and education activities, 8/1/10-7/31/11
[Some of this is a repeat from the year 1 and the year 2 report; new material has also been added]

Research goals, conceptual
Natural selection shapes organisms as integrated sets of traits, but the relative ease with which these traits can be assembled and disassembled in response to selection is contentious. Hormones often underlie the co-expression of traits, and hormonal correlations, like genetic correlations, can promote adaptation or delay evolutionary response. The relative importance of *phenotypic integration and independence* of hormonally mediated traits has significant implications for the evolution of life histories, sexual dimorphism, and population divergence. Integration and independence can be studied via experimental manipulations of hormonal phenotypes, assessment of patterns of natural variation in hormones in relation to phenotype and fitness, comparisons of hormonal phenotypes across populations, and mechanistic studies of hormones and their interaction with target tissues. The research supported by this award employs all these approaches by focusing on the steroid hormone testosterone and its integrating effect on the phenotype of males and females of a songbird species, the dark-eyed junco.

Research goals, chronological
Since 1986 our research group has studied the integrating effect of the steroid hormone testosterone on behavior and physiology in a songbird. Our subject is the Dark-eyed Junco (*Junco hyemalis*), and we study free-living and captive individuals from Virginia, Indiana, California, and South Dakota.

The long-term goal of the research has been to enhance understanding of the evolution of hormone-mediated phenotypes by taking both an experimental and correlative approach to the evolution of life histories. The experimental approach has been to manipulate hormones, testosterone (T) in particular, and then to measure the effects of the manipulation on phenotype and fitness in male and female juncos.

Our focus was the trade-offs between mating and parental behavior and between reproduction and survival, and our approach was to compare the behavior, physiology, and performance of manipulated and control phenotypes. This comparison allowed us to quantify how selection maintains adaptive distributions of phenotypes (e.g., if the manipulated phenotypes have lower fitness than controls) or whether organisms are sometimes hormonally constrained in their ability to respond to selection (if altered phenotypes have higher fitness than controls). The latter possibility might hold, at least in ecological time, because of the tendency of hormones to produce pleiotropic effects, some of which may be disadvantageous if hormones are altered. For example, if males would benefit from higher levels of testosterone, but the consequences for females would be disadvantageous, correlated responses to selection could constrain male evolution.

More recently our approach has been to document individual, sex, and population-level variation in the ability to produce testosterone. We began by assessing variation among males in one population in their tendency to elevate testosterone naturally in response to a standardized upstream hormonal stimulus, a challenge with gonadotropin releasing hormone (GnRH). We then related variability in this capacity to elevate testosterone in response to GnRH to key phenotypic traits, concentrating on traits already known to be affected by experimental elevation of testosterone, and to fitness. We found some very interesting similarities and differences between the phenotypic and fitness consequences of experimentally elevated T and naturally varying ability in the capacity to produce T.

Most recently we have expanded the GnRH challenge approach to females in the first population we studied in Virginia, and to males and females of other junco populations residing in California and South Dakota.

In addition to the GnRH approach, we have begun to compare juncos for a) the hormonal response of females to environmental stimulation (simulated territorial and predator intrusions, b) the response to GnRH of the pituitary as measured by LH in both males and females, and b) the sensitivity to T of target tissues in the brain. The goal is to 'build a map' of sequential links of stimulus and response that gives rise to variation in androgen-mediated traits in the junco. By concentrating effort on environmental triggers, physiological integration, and variability in target sensitivity, we hope to provide a concrete picture of variation in hormone-mediated trait expression that can be compared to both genomic and quantitative genetic understandings of phenotypic variation.

This report summarizes our most recently activities and findings as supported under this award.

Specific objectives

The proposal that led to this award cited four objectives:

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness. Resemblance between males and females in their phenotypic sensitivity to testosterone will be used to predict potential for direct and correlated responses to selection. Traits that respond to elevated testosterone in both sexes but are harmful to one will be interpreted as evidence for constraint. *Ongoing analysis of already collected data will compare testosterone-treated females to controls for extra-pair mating and survival.*

Objective 2: To assess individual variation in hormonal responsiveness and relate that variation to phenotype and fitness. Use of a standardized challenge to the hypothalmo-pituitary-gonadal (HPG) axis with gonadotropin releasing hormone (GnRH) has revealed significant co-variation between natural testosterone levels and mating/ parental effort. Research will extend to females using yolk testosterone as a measure of hormonal phenotype. *Planned studies will relate male response to GnRH to male phenotype and fitness, and yolk T to female phenotype and fitness.*

Objective 3: To compare populations for variation in hormonal responsiveness and testosterone-mediated characters. Suites of hormonally correlated characters may

permit rapid adaptation to new environments; tight linkage of traits to a hormone signal may retard response. *Research will assess degree of phenotypic integration and independence by measuring testosterone response to GnRH and relating it to phenotype in three new populations.*

Objective 4: To assess variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence. Trait evolution involves changes in both hormone signal and target response. *Research will use cellular and molecular techniques to compare neural responses to testosterone and its metabolites in strong and weak responders to GnRH.*

Research training

Under these objectives, the award provided funds to help support **research training** for 2 post-doctoral students (Danielle Whittaker, Kim Rosvall), 8 graduate students (Jonathan Atwell, Christy Bergeon, Kristal Cain, Nicki Gerlach, Dawn O'Neal, Mark Peterson, Dustin Reichard, and Rachel Hanauer).

During 09-10, it supported 6 undergraduates during the academic year (Elizabeth Ansert, Ediri Mitiri, Becky Rice, Elizabeth Swanger, and Meelyn Pandit).

During the summer of 2010, it contributed to the training of 4 REU undergraduate students (Natasha Tonge, Tamara Fetters, Carla Vanderbilt, Meelyn Pandit), and 3 summer field assistants in the period between undergraduate and graduate school during the summer of 10 (Sarah Wanamaker, Matt Boser, Rachel Hanauer), and 1 high school student who will begin as an undergraduate at Indiana U in 2010 (Ingrid Feustel).

During 2010-2011 it helped to train 3 academic year undergraduates (Joe Welkin, Sonya Jayaratna, and Erin Johnson), During summer 2011the award helped to support 3 summer REU students: one primarily supported by a site award to Indiana U (Sonya Jayaratna), one primarily by a site award to the U of Virginia (Cari Lynn Squibb, Virginia Tech University), and one by a supplement to this award (Stephen Fergusen, Wooster College). In addition it helped to support and train 2 summer field assistants (Megan Shreve and Marine Drouilly).

Activities in relation to these specific objectives

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness.

We captured close to 100 females during each of the years 2005, 2006, and 2007 and treated them with subcutaneous implants of T or with empty implants to produce controls. We released them and observed their behavior at the nest (e.g., parental behavior, nest defense) and measured fitness. The implanting phase of this work is over, and our major activity during 2009-10 was to analyze data on relative fitness of the two classes of females, particularly with respect to extra-pair mating. The primary person

engaged in this research was Nicki Gerlach and she completed her thesis and will spend the coming year publishing these data.

During 2010-11, Dr. Gerlach completed the genotyping of offspring produced by T and C-females and produced the first draft of a manuscript describing the fitness consquences of experimentally elevate testosterone. She also gave an oral presentation at the joint meeting of the International Ethological Congress and the Animal Behavior Society in July 2011.

Objective 2: To assess individual variation in hormonal responsiveness and relate that variation to phenotype and fitness.

The objective here has been to quantify variation among individuals in their capacity to elevate testosterone when challenged with a releasing hormone, gonadotropin releasing hormone (GnRH) and to see whether high or low capacity relates to fitness. GnRH ordinarily stimulates the release of luteinizing hormone (LH), which leads to the release of testosterone (T). We captured individuals in the field in Virginia, bled them to determine pre-challenge levels of T, injected (challenged) them with GnRH, collected blood samples 30 min post-challenge, and measured T. We then related the rise in T to attributes of the birds, including parental behavior, aggression, and plumage coloration and immune function.

The critical next step has been to see whether strong or weak responders differ from one another in survival and/or reproduction. Our chief activity in 09-10 was to complete a manuscript which was published in the *American Naturalist* and which reports that selection on T in response to GnRH is stabilizing with respect to survival and slightly but non-significantly directional with respect to reproduction. We have compared these results to those arising out of many years of relating phenotypes induced via experimental elevation of T to fitness. The primary person engaged in this research was former graduate student Joel McGlothlin.

We also documented variation in female juncos in T in response to GnRH and related that variation to aggression at the nest and morphology to determine whether androgynous females have higher or lower fitness as measured by survival and reproductive success. We have found that strong responders to GnRH are also more aggressive towards intruders at the nest. The primary person engaged in this research is Kristal Cain.

During 200-11, Kristal Cain had 3 manuscript published and gave presentation at the International Society for Behavioral Ecology in Australia and the IEC-ABS in Bloomington IN

Objective 3: To compare populations for variation in hormonal responsiveness and testosterone-mediated characters.

During 09-10, we continued to study two populations of juncos derived from southern California, one from the campus of the University of California, San Diego, and one from the nearby Laguna Mountains, both of which were held in a 'common garden' in Bloomington, IN. These populations are of particular interest because they are known to have diverged from one another during the past 25 years after the UCSD campus was colonized in the mid-1980s by juncos believed to have derived from a region that include Laguna Mountain.

We compared the captive birds for seasonal variation in T in response to GnRH, corticosterone in response to handling stress, timing of molt, fattening and Zugunruhe, and behavior, both aggression and boldness. We also compared them for migratory restlessness and fattening. The primary person engaged in this research is graduate student Jonathan Atwell.

We learned that the UCSD birds produce lower peak levels of T but are exposed to T longer because they have a longer breeding season. They are less aggressive, more bold, more parental. They are also less likely to enhance fecundity via extra-pair offspring. In addition, they respond less strongly to stressors, have more robust immune responses and are less susceptible to disease. They also show less nocturnal restlessness during migration and fatten less as well. Thus the colonizing population shows a highly coordinated response to a changed environment, much of which could have been predicted based on their weaker response to GnRH. *It is the ability of a change in one hormonal parameter signal to predict so much about the life history of a newly colonizing population that makes this work so extraordinary.* Atwell has prepared a manuscript summarizing his data from the field and the common garden that will be submitted to Nature in August 2010.

A powerful next step would be to compare juncos that occupy a more challenging environment in terms of novelty or severity of climate to determine whether we can predict the pattern of hormone secretion and associated phenotypic characters prior to study, based on results arising out of comparisons made to date.

During 2010 we also summarized data on T in response to GnRH and aggressive behavior in population of juncos that resides in the Black Hills of South Dakota. We learned that the Black Hills population elevates T more strongly in response to GnRH and is more aggressive towards an intruder as would have been predicted. We did not find within-population variation between T and behavior as we have found in other populations, a result we are still assimilating. The primary person engaged in this research is graduate student Christy Bergeon.

This work continued during 2010-11. Christy Bergeon completed a paired comparison of Virginia and South Dakota juncos to determine how they vary under common circumstances in T in response to GnRH, LH in response to GnRH, and m-RNA expression of aromatase and androgen receptor in key brain regions. These data are currently being analyzed for Christy's Ph.D. thesis.

During 2010-11, the California project also moved forward as Jonathan Atwell completed his Ph.D thesis and the summary comparing the two populations in the field and in a common garden. He submitted his findings to Nature, Science, and PNAS, because we thought they merited a venue of that order, but the journals said no, so Atwell is in revising the manuscript.

Objective 4: To assess variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence.

We made very strong progress towards this objective during 2010. First, two people associated with the award, Chirsty Bergeon Burns and Kim Rosvall, visited the laboratory of Barney Schlinger at UCLA to learn micro-dissection and quantitative PCR. Dr. Rosvall then collected male and female juncos in Virginia after assessing their behavioral response to an intruder, and she used the qPCR method to quantify mRNA for androgen receptor (AR), estrogen receptor (ER) and aromatase (AROM) in 2 brain regions, hypothalamus and medial amygdala. She found significant correlated individual variation in brain and behavior in the field (!). This same brain tissue will also be surveyed for receptor density using immunocytochemistry in the coming year.

Christy Bergeon Burns conducted a study to determine which links in the hypothalamic-pituitary-gonadal (H-P-G) axis account for individual variation in the production of testosterone, i.e., do individuals vary more in the degree to which they elevate LH in response to GnRH or T in response to LH? Burns brought juncos from South Dakota to Bloomington, held them over the winter, and measured their response to GnRH in terms of both LH and T. She found individual variation at each level that was correlated with the production of T. She will next compare the gonadal tissues for AR, ER and aromatase. She is also in the process of collecting a series of individuals from Virginia, which will allow a geographic comparison in the coming year.

During 2010-2011, the progress was also rapid. Dr. Rosvall and Christy Burns perfected the qPCR to allow them to quantify AROM, AR and, most recently with new primers, ER. They trained students to help and burned through many samples. They have shown, for example, co-variation in the intensity of aggressive behavior and the quantity of AROM and AR in the hypothalamus and the nucleus teniae. They also compared the sexes for response to a territorial intrusion and found striking similarities.

During he field season of 2011 Dr. Rosvall compared the impact of brief and prolonged behavioral challenges on the transcriptional responses of key brain regions of birds observed and captured in the field.

Dr. Rosvall was a symposium speaker at the annual meeting of the Society for Behavioral Neuroendocrionology and presented her newest data, and Ms Burns gave a presentation at the joint IEC-ABS meeting in July on her latest data.

As part of objective 4 and with additional funding we have begun to compare gene expression in male and female juncos treated with testosterone.

Additional research and educational activities not stated in the award proposal that have been facilitated because of the existence of NSF funding.

1) *Seasonal, sexual, and population- and species-level variation in volatile compounds found in preen oil*. Using HPLC, we compared the volatile composition of preen oil in juncos by sex, season and population and in an array of species breeding in southern Indiana. We also measured the impact of preen oil on incubation behavior and laid the groundwork to relate variation in preen oil composition to variation in the major histocompatibility complex (MHC). We are collaborating with Milos Novotny and Helena Soini. The primary person engaged in this research is post-doctoral student Danielle Whittaker and she published 2 papers during 2009 and 2010 and two more papers in 2011

2) *Junco transcriptome.* We have produced a compiled and partially annotated transcriptome for the junco. We are collaborating with John Colburne of the IU Center for Genomics and Bioinformatics. The primary person engaged in this research is graduate student Mark Peterson, and the annotation was completed in 2011. The results are in manuscript form and will soon be submitted to GMC genomics. The transcriptome was used to generate a gene array and we are using it to compare impacts of testosterone on gene expression.

3) *Documentary film of junco research.* We have begun to produce an engaging educational multimedia project that will serve to enhance public understanding of evolution and also serve to document our past and current research on the junco. The junco is a classic species for studies of speciation and environmental physiology and is also the subject of our ongoing research on the evolution of complex (hormone-mediated) phenotypes, the expression of aggressive and parental behavior, sex differences in migratory behavior, and the responses of bird populations to climate change and urbanization. During 2010 we collected footage in California, Guatemala, and Mexico, including Guadalupe Island. The primary person engaged in this project is Jonathan Atwell and videographer Steve Burns, along with graduate student Christy Bergeon Burns.

   During 2011 the film crew went to Baja Sur at the southern tip of Baja to film an endemic junco and made filming expeditions to San Diego and to Teton National Park in Wyoming. The footage has been documented and the real work has begun. This project is now funded by an OPUS proposal but began under this award.

4) *Vocal behavior in the dark-eyed junco and its potential role in population divergence.* Juncos produce two types of song. One is the loud territorial song that it is referred to as long range song (LRS). The other type is produced at low volume, is complex, is sung during courtship, and is referred to as short-range song (SRS). The territorial song is quite similar geographically, and males in the eastern US respond equally

strongly to playbacks of eastern and western LRS (unpublished data). Much less is known about the 'meaning' of SRS, whether it varies geographically, and how it impacts female reproductive physiology. We are comparing the structure and function of LRS and SRS within and among populations of juncos and during 2010 we began to collect recordings from a new population of juncos, the pink-sided junco in Wyoming.

We are also using miniature transmitting microphones to track the vocal behavior of free-ranging juncos in collaboration with Dr. Dave Enstrom of the U of Illinois.

During 2011 we wrote up the first paper summarizing differential responses of male juncos to playback of long and short range song, a paper that will appear in American Naturalist.

The primary person engaged in this project is graduate student Dustin Reichard.

5) *Migratory behavior in the dark-eyed junco.* Juncos differ in the distance they migrate in autumn by sex, and females migrate farther than males. We have asked whether recent climate warming has had an influence of migration distance and our data show that female juncos have apparently shortened their migrations, perhaps in response to climate warming. We had anticipated that immune function might vary geographically or as a function of competition between the sexes but did not find support for this hypothesis. The primary person engaged in this project was graduate student, Dawn O'Neal, who completed her Ph.D on this project in 2010.

   In 2010-2011 we participated in a workshop that attracted legal scholars, conservation biologists and migration biologists. We discussed how best to preserve migrations and published the results of our findings in an environmental law journal.

   Jonathan Atwell was the lead on this project.