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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).

PROJECT SUMMARY

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. In this proposal, we employ all these approaches, 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. We have 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.*

Intellectual merit.—Research described here will make important contributions to our knowledge of the evolution of hormonally mediated phenotypes. By focusing on the effects of one integrating, signaling molecule, testosterone, and one species that has received intensive, long-term study, the work will address the interacting effects of direct and correlated responses to selection. In terms of areas identified by the Workshop on Frontiers in Evolutionary Biology (NSF, 2005), the research should advance understanding of the evolution of integrated phenotypes and of adaptive divergence of populations.

Broader impacts.—Research described here will provide opportunities for training future scientists of diverse backgrounds in the conduct of laboratory and field research that combines techniques and perspectives from multiple disciplines. Undergraduates in particular will benefit from exposure to life at a field station and the opportunity to encounter faculty and like-minded students from other institutions, and to cope with the challenge of ever changing conditions in the field. We make a point of addressing ethical issues associated with the responsible conduct of science, including data quality, animal welfare, regulatory compliance, sharing of credit, and dissemination of results. Potential societal implications of the research include relevance to the study of (1) hormonally active agents in the environment, and (2) response of natural populations to altered environments.

PROJECT DESCRIPTION

This proposal seeks funding to continue a long-term research program addressing the **role of hormones in the evolution of life histories, sexual dimorphism, and phenotypic integration.** Central to the research are the hormone testosterone (T) and a songbird species, the dark-eyed junco (*Junco hyemalis*). The research focuses on the expression and evolution of testosterone-mediated characters by male and female juncos within and among populations. Results of the research will bear on general issues of adaptation and constraint as reflected in phenotypic integration and independence.

RELATIONSHIP OF PROPOSED RESEARCH TO PAST RESEARCH

Progress has been made in overlapping stages. *The first stage* involved experimental manipulation of male phenotypes using testosterone (T) implants. The goals were 1) to identify phenotypic characters that were enhanced, suppressed, or left unchanged by elevation of testosterone and 2) to determine the net effect of any alterations on fitness. While acknowledging that many of the hormones effects were probably indirect, we concluded that testosterone plays a key role in trade-offs between mating effort and parental effort and between reproductive effort and self-maintenance.

During *the second stage* we conducted similar studies on females with the goal of comparing the sexes for their sensitivity to testosterone in order to predict how co-evolution of the sexes might proceed. These studies, nearing conclusion, suggest that elevated testosterone can be highly costly to females.

The third stage relates individual variation in male testosterone to phenotypic characters identified as hormone-sensitive in the first stage. Variation is measured by the levels of testosterone released in response to stimulation of the hypothalmo-pituitary-gonadal axis (HPG) when challenged with gonadotropin releasing hormone (GnRH). The goals are 1) to quantify natural variation in testosterone and testosterone-mediated characters and 2) to explore how selection might build integrated phenotypes through direct and correlational selection. Continuing research will relate natural variation in T to male fitness and apply the same approach to females.

The fourth stage added a new dimension to the research by comparing testosterone levels across populations of juncos at new study sites in California and South Dakota. Here the goal is to enhance understanding of the role played by hormones such as testosterone in population divergence.

The fifth stage will address phenotypic targets of testosterone. To date, manipulations and observations have been based entirely on experimental and natural variation in signal strength, i.e., plasma levels of testosterone. In this proposal we describe our initial efforts to explore variation in target tissue response to testosterone by measuring receptor density and mRNA expression. Proposed research will focus on males within a single population. Future research will compare males to females within populations and also compare males to males across populations.

RESULTS OF MOST RECENT PRIOR NSF SUPPORT

NSF BSC 05-19211, $372,756 + 2 REU supplements, $12,000, 1 June 2005 – 31 July 2008.
Title: *Testosterone in female songbirds: natural, sexual, and correlated responses to selection.*
Contribution to the development of human resources.—Students receiving training with the help of BSC 05-19211: **2 post-doctoral associates** (2 female), **10 graduate students** (4 male, 6 female, 1 African-American), **16 undergraduate or post-undergraduate students** (5 male, 11 female, includes 1 African-American, 9 REUs to this award or to Mountain Lake Biological Station). Of these, 7 have entered graduate school or are in the process of applying, 5 are still undergraduates, and 4 are co-authors on publications or manuscripts.

Post-doctoral associates: J Jawor (now Asst. Prof., Southern Mississippi U), D Whittaker (still in training, SiT). ***Graduate students***: J Atwell (SiT), C Bergeon (SiT), K Cain (SiT), N Gerlach (SiT), T Greives (SiT), B Heidinger (now post-doc, U of Glasgow, Scotland), J McGlothlin (now post-doc, U Virginia), D O'Neal (SiT), D Reichard (SiT), S Schrock (completed M.A.), D. Zysling (SiT). **Undergraduate students and field assistants**: K Ainsworth, O Ali, A Bessler, S Campbell-Nelson, B Houdek, C Knox, A Lindsay, K Pavlis, J Phillips, D Reichard, K Robertson, B Schultz, E Spevak, P Stevens, E Swanger, R Young.

Publications resulting from the NSF award, 2005-2008.—**Total is 19**, includes 2 under review and 1 about to be submitted. **Listed here are the 10 most directly related to objectives of 05-19211.**

O'Neal, DM, *Reichard, DG, *Pavilis, K and ED Ketterson. 200x. Effects of experimentally elevated testosterone on female parental care in dark-eyed juncos. To be submitted to *Animal Behaviour.*

McGlothlin, JW and ED Ketterson. 2008. Hormones and the continuum between adaptation and constraint. *Phil. Trans. Royal Society,* published on line. doi:10.1098/rstb.2007.0002

McGlothlin, JW, Jawor, JM, Greives, TJ, Casto, JM, *Phillips, JL, and ED Ketterson. 2008. Hormones and honest signals: males with largest ornaments elevate testosterone more when challenged. *Journal of Evolutionary Biology*, published on line doi:10.1111/j.1420-9101.2007.01471.x

McGlothlin, JW, Jawor, JM, and ED Ketterson. 2007b. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. *American Naturalist*, 170: 864-875. doi: 10.1086/522838

McGlothlin, JW, Duffy, DL, *Henry, JL, and ED Ketterson. 2007a. Diet quality affects an attractive white plumage pattern in dark-eyed juncos (*Junco hyemalis*). *Behavioral Ecology and Sociobiology* 61:1391-1399. doi:10.1007/s00265-007-0370-x

Jawor, JM, McGlothlin, JW, Casto, JM, Greives, TJ, Snajdr, E, Bentley, GE, and ED Ketterson. 2007. Testosterone response to GnRH in a female songbird varies with stage of reproduction: implications for adult behaviour and maternal effects. *Functional Ecology* 21:767-775.

Jawor, JM, McGlothlin, JW, Casto, JM, Grieves, TJ, Bentley, G, Snajdr, EA, and ED Ketterson. 2006. Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis). General and Comparative Endocrinology,* 149:182-189. doi:10.1016/j.ygcen.2006.05.013

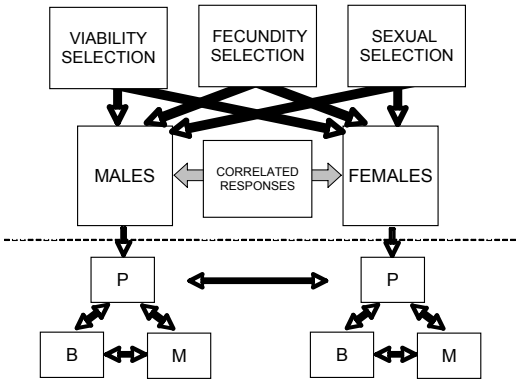
Greives, TJ, McGlothlin, JW, Jawor, JM, Demas, GE, and ED Ketterson. 2006. Testosterone and innate immune function inversely covary in a wild population of breeding dark-eyed juncos (*Junco hyemalis*). *Functional Ecology* 20: 812-818. doi:10.1111/j.1365-2435.2006.01167.x

Zysling, DA, *Greives, T, Breuner, C, Casto, JM, Demas, GE, and ED Ketterson. 2006. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis carolinensis). Hormones and Behavior* 50(2): 200-207. doi:10.1016/j.yhbeh.2006.03.004

Jawor, JM, *Young, R, and ED Ketterson. 2006. Females competing to reproduce: dominance matters but testosterone may not. *Hormones and Behavior,* 49 (3): 362-368. doi:10.1016/j.yhbeh.2005.08.009

**Indicates authorship by an undergraduate student (n=6).*

INTRODUCTION



Research described in this proposal seeks to relate selective networks to proximate mechanisms that give rise to phenotypic expression by focusing on the 'hormone in the middle.' The upper half of Figure 1 depicts how differences in the relative strengths of viability, fecundity and sexual selection can give rise to sex differences. Correlated responses to selection may serve to maintain similarities between males and females despite selective pressures that might cause them to be different. The boxes and arrows below the dotted line depict the multiple morphological (M), behavioral (B), and physiological (P) traits that contribute to variability within each sex and to differences between them.

When these multiple traits are correlated in their expression, they give rise to within-sex syndromes and to phenotypes that vary by sex (Sih et al. 2004; Ketterson et al. 2005).

The hormone testosterone is of particular interest because of its ability to 'orchestrate' suites of traits and account for sex differences. That is, testosterone has multiple phenotypic effects and can thus be seen as analogous to a gene with multiple pleiotropic effects (*hormonal pleiotropy*)(Ketterson and Nolan 1999; Zera et al. 2007; Williams 2008). Testosterone also interacts with other hormones in a manner analogous to epistatic interactions among genes (*physiological epistatsis*)(Cheverud and Routman 1995; Goodson 2005; McGlothlin and Ketterson 2008). And, to the extent that hormonal correlations among traits are analogous to genetic correlations, testosterone's multiple effects can give rise to phenotypic integration via correlational selection (Finch and Rose 1995; McGlothlin and Ketterson 2008).

The concepts of hormonal pleiotropy, physiological epistasis and hormonal correlations, as well as the conceptual framework laid out in Figure 1, raise many unanswered questions. **One important question is the functional importance of hormonal correlations between the sexes.** Which aspects of the female phenotype, for example, are sensitive to testosterone, and might their existence serve to favor compromises in males between the advantages of testosterone-mediated characters and costs of such characters in females? We have addressed this question experimentally by elevating testosterone in females and documenting the phenotypic effects (Clotfelter et al. 2004; Ketterson et al. 2005; Zysling et al. 2006). Ongoing studies proposed here will demonstrate the long-term effect of these changes on fitness.

A second important question is the nature of individual variation in hormonal systems and how that variation relates to the evolution of phenotypes. At issue is the degree to which variation in a hormone signal can explain variation among individuals. Endocrinologists have remained skeptical about the predictive power of hormone-phenotype relationships (Adkins-Regan 2005; Hau 2007; Ball and Balthazart 2008; Williams 2008), and understandably so. Hormones are inherently variable and 'intended' to permit plastic responses to changing environments. On the other hand, components of the endocrine system such as the responsiveness to stimulation of the hypothalamo-pituitary-adrenal-axis have proved responsive to artificial selection (Evans et al. 2006) and to natural selection in the wild (Blas et al. 2007), demonstrating the evolutionary importance of variation in the strength of the hormone signal.

Hormonally mediated characters can clearly evolve owing to components of the endocrine system other than hormone secretion, such as hormonal affinity for carrier proteins, tendency towards degradation or conversion, and ability to bind with target tissues (Nijhout 2003, Nelson 2005). **Critically, very little is known about whether these components tend to vary independently or in tandem, and thus whether hormones promote phenotypic integration or allow for phenotypic independence.** Consequently, when individuals differ in the expression of hormonally mediated characters, we rarely know whether to attribute the difference to variation in signal strength, target sensitivity, or both (Silverin et al. 2004; Canoine et al. 2007; Hau 2007).

Further, little is known about which of these components evolves most readily to allow for diversification of hormone-mediated characters over time and space. Thus, if a population enters a new environment that leads to altered expression of hormonally mediated characters, is that alteration likely to have come about through changes in hormone secretion (signal strength) or changes in response to a fixed level of secretion (target tissue sensitivity)? If the phenotype is tightly integrated and only the signal responds, adaptive modification may be limited by **phenotypic integration**. If individual target tissues readily 'unplug' from a hormone signal in response to selection, then the phenotype may be seen as a loose confederation that responds on a trait-by-trait basis. In that case adaptive modification may proceed by **phenotypic independence** (McGlothlin and Ketterson 2008).

The degree to which adaptive divergence may be facilitated or retarded by suites of hormonally correlated

characters has become an area of contention (Hau 2007; Adkins-Regan 2008; Lessells 2008; McGlothlin and Ketterson 2008). At one extreme is the '*evolutionary constraint hypothesis*,' which "assumes that testosterone signaling mechanisms and male traits evolve as a unit," and that "the actions of testosterone are similar across sexes and species, and only the levels of circulating testosterone concentrations change during evolution" (Hau 2007). In contrast, the '*evolutionary potential hypothesis*,' proposes that the "linkage between hormone and traits itself can be shaped by selection" (Hau 2007). While we prefer to use different terminology, namely *phenotypic integration* and *phenotypic independence*, we anticipate that in natural populations both signal and target must evolve. Studies proposed here assess the relative importance of integration and independence by comparing natural populations of a well studied bird.

RESEARCH OBJECTIVES

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness. Knowledge of the extent of resemblance between the sexes in their phenotypic sensitivity to testosterone can be used to predict the potential for direct and correlated responses to selection. Traits that are sensitive to an experimental increase in T in only one sex should not be subject to correlated responses in phenotype. Traits that are sensitive in both sexes, but beneficial in only one, can provide evidence for constraint.

Completed experiments by us on males and females have shown similar effects in both sexes of experimentally elevated T on aggression, immune function, and nest defense. Parental behavior, on the other hand, is sensitive to T in males but largely insensitive in females. In earlier experiments we demonstrated higher fitness in T-treated males than in male controls; data to date predict significant fitness costs to T-treated females in terms of reduced fecundity. *Ongoing analysis of previously collected data will compare the effect of testosterone on extra-pair mating and survival in females*.

Objective 2: To assess individual variation in hormonal responsiveness and relate that variation to phenotype and fitness. Knowledge of natural variation in testosterone is clearly critical to understanding its role in phenotypic evolution, co-evolution of the sexes, and population divergence. Completed studies in one population have shown that male juncos and fertile females vary in how much they elevate testosterone in response to a standardized injection of gonadotropin releasing hormone (GnRH). In males, the response to a GnRH challenge is repeatable. It also co-varies with significant phenotypic characters: strong responders are more aggressive, more ornamented, and less parental, as would be predicted by a trade-off between mating effort and parental effort. In females, response to GnRH co-varies with testosterone in her eggs. *Planned studies will relate yolk T to female phenotype and response to GnRH in both sexes to fitness so as to assess how selection acts on natural variation in testosterone.*

Objective 3: To compare populations for variation in hormonal responsiveness and in testosterone-mediated characters. Knowledge of natural variation in hormones among populations is critical to understanding the role of hormones in population divergence. Under the hypothesis of *phenotypic integration*, quantitative traits that co-vary with T in one population are predicted to show similar relationships in other populations. Under the hypothesis of *phenotypic independence,* relationships between hormones and traits will be labile. Further, when comparing a new population to a focal population, integration predicts that if the new population exhibits higher levels of traits that were correlated with response to GnRH in the focal population, then members of the new population should also show stronger responses to GnRH than the focal population. Uncoupling of these relationships would indicate varying levels of phenotypic independence. *Research conducted under this proposal will test these predictions by measuring patterns of T in response to GnRH and phenotype in three additional populations and comparing results to those already obtained.*

Objective 4: To assess the variation in target tissue sensitivity to testosterone in relation to phenotypic integration and independence. Knowledge of evolutionary implications of testosterone's

effect on the phenotype will be incomplete until we know more about how target tissues interact with T. *Research conducted under this proposal will decompose individual variation of T in response to GnRH by asking 1) whether, as phenotypic integration would predict, strong responders to GnRH are more responsive along the length of the hypothalmo-pituitary-gonadal (HPG) axis, and 2) whether strong responders also exhibit higher levels of steroid enzymes and receptors in target tissues.*

RELATION TO PRESENT STATE OF KNOWLEDGE IN THE FIELD

Objective 1: Testosterone in females. In males, the phenotypic effects of testosterone have been well characterized, as has the likely effect of elevation in T on viability, fecundity, and mating success (Klein et al. 1997; Ketterson et al. 1999; Wingfield et al. 2001; Westneat et al. 2003; Reed et al. 2006). Females also produce testosterone, but until recently its effects on the female phenotype have been less well documented (Staub and De Beer 1997; Van Duyse et al. 2002; Ketterson et al. 2005; Moller et al. 2005; Mank 2007). Interest in female testosterone has, however, been growing rapidly (Gill et al. 2007; Peters 2007; Sandell 2007). Studies relating experimentally elevated or natural levels of female testosterone to fitness remain to be done.

Objective 2: Individual variation in hormones, hormonally mediated characters, and fitness. Behavioral ecologists and evolutionary endocrinologists often treat hormones like quantitative traits that co-vary with trait expression (Zera et al. 2007; Williams 2008) and are subject to modes of analysis developed for quantitative characters (McGlothlin and Ketterson 2008). This view derives support from artificial-selection studies in which selection on hormone levels can alter phenotype, and selection on hormonally mediated phenotypes can alter hormone levels (Gross and Siegel 2000; for more examples, see Zera 2007; Williams 2008).

In contrast, the prevailing view among endocrinologists has been that plasma hormones are 'permissive.' In this view, once hormone concentration exceeds a threshold, further elevation of the hormone does not enhance trait expression (Adkins-Regan 2005; Adkins-Regan 2008; Ball and Balthazart 2008; Williams 2008). Even apparent co-variation between hormone levels and phenotype can be explained by supposing individual variation in thresholds (Hews and Moore 1997). This view derives support from 'negative' data in which hormone levels and phenotype have not been correlated (Adkins-Regan 2005), and from studies of castrates in which the same dose of hormone has elicited very different behavioral responses (Ball and Balthazart 2008). The middle ground is occupied by theory. If correlational selection favors particular combinations of traits, then it should favor mechanisms that give rise to them, predicting co-variation between signal strength and target sensitivity if (McGlothlin and Ketterson 2008). Clearly much remains to be learned.

Objective 3: Variation in hormones and hormonally mediated characters among populations. Debate surrounds the question of whether adaptive divergence can be retarded by hormonal mediation of suites of characters (Hau 2007; Adkins-Regan 2008; Lessells 2008; McGlothlin and Ketterson 2008). On the one hand is incontrovertible evidence that ancestral signal-target relationships can be overcome; witness the female spotted hyena's enlarged phallus (Frank 1997). Alternative arguments and theory based in quantitative genetics note the retarding effect of strong genetic (and thus perhaps hormonal) correlations on evolutionary outcomes (McGlothlin and Ketterson 2008). In this view, phenotypic correlations that are built up through adaptive correlational selection in one environment may prove at least a temporary impediment to an adaptive evolutionary response to a changed environment. Hau (2007) recommended that we approach these issues by comparing subspecies or populations. Because evolutionary endocrinology is such a young discipline (Zera et al. 2007), relatively few direct comparisons of hormonally mediated characters and their underlying mechanisms have been made across populations (but see studies of hormones and breeding phenology, e.g., Moore et al. 2005). Such comparisons should prove fruitful.

Objective 4: Variation in target tissue sensitivity to testosterone.
Two early generalizations in avian environmental endocrinology were 1) males elevate testosterone naturally when challenged by an intruder, and 2) experimental elevation of testosterone suppresses male parental behavior. Recent studies have discovered examples of male *insensitivity* to social challenge (Goymann 2007) and to experimentally elevated testosterone (Lynn et al. 2002). These exceptions have been explained in ultimate terms, e.g., failure to respond to a social challenge when there is little opportunity for extra-pair mating (Goymann 2007), or to T-implants when parental care is essential to offspring survival (Lynn et al. 2002). More knowledge is needed about the mechanisms underlying variation in sensitivity at multiple levels. Fortunately, quantitative methods for assessing sensitivity can be employed in service of these questions (Ball and Balthazart 2008). Immunocytochemistry (ICC), for example, can detect variation in density of hormone receptors, and *in situ* hybridization can detect variation in mRNA for receptors and steroid metabolizing enzymes (Silverin et al. 2004; Forlano et al. 2006; Ball and Balthazart 2008).

Two recent studies have used *in situ* hybridization to compare steroid sensitivity in key brain nuclei. Canoine et al. (2007) compared spotted antbirds by season and found that circulating steroids declined in the non-breeding season, but brain sensitivity to hormones increased. In particular, mRNA expression of androgen and estrogen$_\alpha$ receptor (AR, ER$_\alpha$), and the enzyme aromatase (which converts testosterone to estradiol) varied by season: ER$_\alpha$ expression was greater during the non-breeding season in the pre-optic area (POM), as was AR expression in the nucleus taeniae (nT), which is associated with aggression. These findings were interpreted as evidence for increased sensitivity to steroids in the regulation or reproductive and aggressive behavior, results that suggest independence of signal and target expression. A second important study compared mRNA expression in black coucals, a polyandrous African bird species that exhibits 'sex role reversal.' Female black coucals are more aggressive and less parental than males, and exhibit greater AR mRNA expression in the (nT) (Voigt and Goymann 2007). These results (and others) suggest that testosterone may mediate aggression in female black coucals via greater target sensitivity, and in males via a stronger signal, further evidence for phenotypic independence.

STUDY SYSTEM AND GENERAL METHODS

Study species.—The dark-eyed junco (*Junco hyemalis*) is an abundant songbird throughout North America (e.g., Deviche et al. 2000; Nolan et al. 2002; Meddle et al. 2006; Mila et al. 2007). Juncos are quantitatively dimorphic in body size and amount of white in the outer tail feathers (tail white), a trait that enhances male but not female attractiveness (Hill et al. 1999; Wolf et al. 2004; McGlothlin et al. 2005). The sexes differ qualitatively in vocal behavior (typically only males sing) and parental behavior (only females incubate). Juncos are territorial when breeding and form socially monogamous bonds, but frequently produce young via extra-pair fertilizations (~24%)(Ketterson et al. 1997; Raouf et al. 1997; Reed et al. 2006). Females build the nest and incubate eggs (clutch size, 3-4; brood number, 1-3); both sexes care for nestlings and fledglings. Nest predation is common in most years; site fidelity is nearly complete among males, less so among females, and least among offspring (~15%).

Study subspecies.— We have traditionally studied the Carolina junco, *J.h.carolinensis* in Virginia (VA). Work in the present proposal adds populations in California (CA) and South Dakota (SoDA). In CA, we will focus on two populations of *J.h.thurberi* that have been the subject of recent and important studies by Price and his students (Rasner et al. 2004; Yeh 2004; Yeh and Price 2004; Price et al. 2008). In SoDA we will study the white-winged junco *J.h. aikeni*, an endemic to the Black Hills, which is distinctive in being the subspecies of junco that has the largest body size and the most white in the tail (Nolan et al. 2002).

Field sites.—We have traditionally worked at Mountain Lake Biological Station (University of Virginia), near Pembroke VA. We use same net locations and standard procedures each year, censusing the population twice, once from late April to mid-May, and again from mid-July to early August. Since 2005 we have studied (with Trevor Price) the CA populations on the campus of the U of California San Diego

(UCSD) and a nearby (~70km) site in the Laguna Mountains. Beginning in 2005, then again in 2007, we initiated a study of the SoDA juncos at sites near Custer, SD, where juncos are extremely abundant.

Study site for captives.—When studying captive juncos, we house them either at the biological station in Virginia or in Indiana. In IN we have a temperature- and day-length-regulated 11-room indoor aviary d an outdoor aviary that can be divided into 30 breeding compartments or serve as a single enclosed space. Juncos thrive in captivity as evidenced by high survivorship and excellent physical condition.

Determining circulating levels of plasma and yolk hormones.—To detect steroid hormones in plasma and egg yolk [P, T, DHT, E2 and corticosterone (CORT)], we use tritium-based radio-immunoassays (RIAs) as introduced by Wingfield and Farner (1975). We also use RIA to measure CORT directly on extracted plasma (Clotfelter et al. 2004; Zysling et al. 2006). For most measurements of plasma testosterone we use enzyme immunoassays (EIAs, Assay Designs, Inc., #901-065) (Clotfelter et al. 2004; Jawor et al. 2006; McGlothlin et al. 2007), employing a kit that that equals RIAs in terms of inter- and intra-assay variation, can be conducted in far less time, and, most importantly, requires smaller volumes of plasma, which is essential for the repeated sampling that is part of the GnRH challenges we employ.

Manipulating T in females.— To elevate T in females experimentally, we used silastic implants (1.5 mm i.d., 2.0 mm o.d.), employing smaller doses than used with males (one 5mm implant vs. two 10mm implants in males). Controls received empty implants. We assigned treatment at random, and kept treatment constant in females that returned in successive years. Impact of implants on plasma T in females has been assessed multiple times (e.g., Clotfelter et al. 2004; Zysling et al. 2006) and shown to induce levels that closely resemble the early season, natural female peak. Implants do not affect circulating levels of estradiol (unpublished data); they do elevate CORT and CORT binding globulin (Clotfelter et al. 2004; McGlothlin et al. 2004; Zysling et al. 2006).

Fitness measures: extra-pair mating/survivorship.—We routinely collect blood samples from adults and offspring for paternity analysis. We extract DNA using standard phenol/chloroform methods. Ten variable dinucleotide microsatellite loci [Dpu01 (14 alleles), Dpu16 (13 alleles), GF01b (14 alleles), GF05 (13 alleles), GF06 (5 alleles), GF14 (14 alleles), A03 (13 alleles), C08 (14 alleles), G08 (10 alleles), and Ju05 (9 alleles)] are amplified using fluorescently labeled primers in 10µl multiplex PCR reactions (5 loci per multiplex) using a Qiagen multiplexing kit and a protocol modified for juncos by Price and T Harr (U of Chicago/Max Planck). Fragments are sized using the ABI3730 sequencer and GeneMapper software. Each individual is genotyped a minimum of two times to verify alleles. Paternity is assigned using the software CERVUS (Marshall et al. 1998), which employs a likelihood-based algorithm that takes genotyping error into account. Regarding fecundity and viability, we document nest success, year-to-year site fidelity, mate fidelity, and survivorship using MARK (O'Neal et al. 200x; Burnham and Anderson 2002; Reed et al. 2006).

Measuring T in response to GnRH.— To assess natural variation in the maximum response of the HPG axis, we challenge with gonadotropin releasing hormone, GnRH, which stimulates release of LH, which in turn stimulates release of testosterone (T) by the gonads. To conduct a GnRH challenge we collect an initial blood sample (~100 µl) from the wing vein (*initial T*), inject the left pectoral muscle with 50 µL of a solution containing 1.25 µg of chicken GnRH-I (Sigma L0637; American Peptide 54-8-23) dissolved in 0.1 M phosphate-buffered saline solution into the left pectoral muscle and place the bird in an opaque bag. Exactly 30 minutes after the injection, we take a second (~100 ul) blood sample to measure *post-challenge T*. Blood samples are centrifuged, and the plasma fraction is reserved and frozen until assayed (see above). The difference between initial T and post-challenge T is computed as the *rise in T in response to GnRH* (Jawor et al. 2006). Importantly, testosterone levels produced after a GnRH challenge are repeatable (Jawor et al. 2006) and correlated with natural increases in testosterone produced in response to a simulated territorial intrusion (McGlothlin et al. 2008).

Other methods.—Our group has successfully used all the methods proposed for the experiments described below except those associated with Objective 4 (assessing target tissue sensitivity), which we will describe below. Well-tested methods include bleeding nestlings and adults for hormones and for DNA, performing steroid RIAs on plasma (e.g., Schoech et al. 1998) and egg yolks (Lipar and Ketterson 1998; Casto et al. 1999) and EIAs on plasma (Clotfelter et al. 2004), video taping at nests (Clotfelter et al. 2004), quantifying variation in plumage coloration (tail-white) using image analysis (Wolf et al. 2004; McGlothlin et al. 2008), measuring immune function as cell-mediated (e.g., swelling in response to PHA), humoral (e.g., response to antigen challenge) or innate (e.g., complement)(Casto et al. 2001; Greives et al. 2006; Zysling et al. 2006), measuring sperm density in the field (Kast et al. 1998), and measuring aggressive behavior in response to a stimulated territorial intrusion (McGlothlin et al. 2008). We have many years experience monitoring nests and eggs and nestlings (egg size/body mass/linear dimensions). We have successfully transported live juncos from Virginia to Indiana by car and by plane from Canada and California.

To summarize, males and females resemble one another naturally in their testosterone profiles, manipulations of T in females induce levels seen naturally in early spring (Ketterson et al. 2005), and females are not harmed by implants (Clotfelter et al. 2004). Males and females vary among themselves in the degree to which they elevate T in response to GnRH, and in males that elevation co-varies with measures of mating effort and parental effort (Jawor et al. 2007; McGlothlin et al. 2007; McGlothlin et al. 2008). T in response to GnRH also varies among populations and the logistics for further study have been addressed (Atwell et al. in prep. Cain et al. in prep., see below).

PROPOSED STUDIES

Objective 1: To relate sensitivity to experimentally elevated testosterone in females to male sensitivity and to fitness. *Proposed research will complete analysis of experimental studies relating testosterone to female phenotype and fitness.*

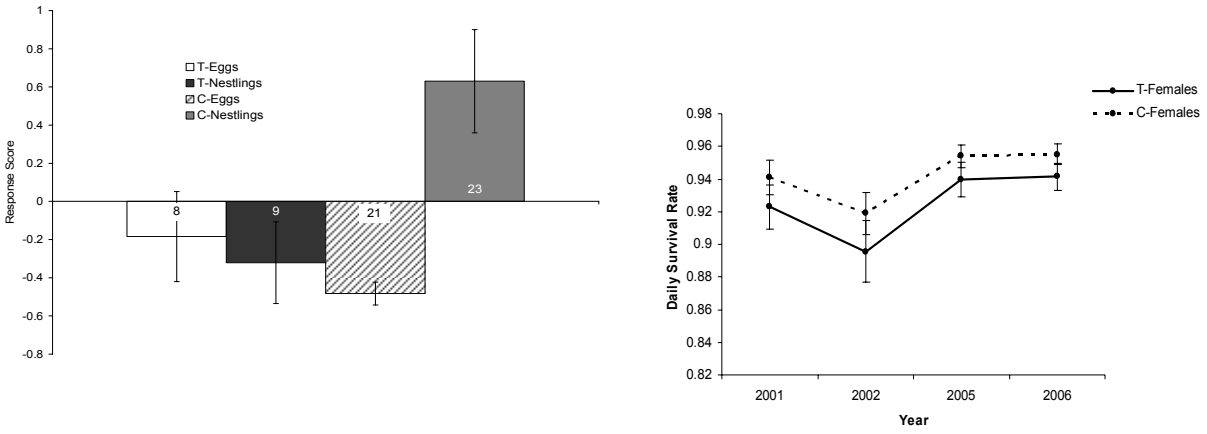
Rationale.— Results of implant studies indicated that male juncos with higher-than-normal levels of testosterone should out-compete those with typical levels owing to greater extra-pair mating success (Reed et al. 2006). The fact that higher T-levels have not evolved led to the 'constraint hypothesis', which posited that phenotypic, and potentially genetic, correlations between the sexes might retard the evolution of higher male testosterone. To address this alternative, we elevated testosterone experimentally in females and asked whether T-treated females exhibited male-typical traits, and, if so, whether these traits put them at a fitness disadvantage. To date it appears that higher testosterone is indeed detrimental to females, providing at least provisional support for the constraint hypothesis, but analysis is still underway.

Progress to date.— Following a two-year study conducted in 2001-02 in VA (Clotfelter et al. 2004), we again implanted females during 2005-2007, 125 with testosterone (T-females) and 142 as controls (C-females). The implanting phase of the research, now over, has shown that T enhances female aggression, suppresses immune function, and elevates corticosterone and corticosterone binding globulin (Zysling et al. 2006). T has no detectable effect on incubation or defense of eggs against predators, suggesting that unlike in males, female parental behavior is insensitive to experimentally elevated T (Clotfelter et al. 2004). Recently, however, we found that while T does not suppress nestling feeding, it does suppress nest defense during the nestling period (O'Neal et al. 200x)(see Figure 2, left below).

The important task that remains is to complete analysis and publication of data collected from the field, comparing the sexes for fecundity, survival, and mating success. The strongest determinants of fecundity are likely to be whether a female initiates a nest and how successful she is at escaping nest predators. In 2005-06, we found nests for 49% of T-females (39 of 80) vs. 66% of C-females (64 of

97)(chi-square, $p < 0.003$), which suggests that treatment with T suppresses the initiation of reproduction, but notably not in all females. *Females apparently vary in their sensitivity to T, suggesting that for a subset of the females T was inhibitory.* For those that laid eggs, clutch size did not differ. *Nest success appears to be suppressed by testosterone treatment* ($P < 0.06$, analysis still underway, Figure 3, right below). Regarding extra-pair parentage, based on very preliminary data (N Gerlach in prep.) from one year, T-treated females produced fewer extra-pair offspring (EPO) than controls, but not significantly so (2 EPO of 14 in T-females, 14.3% vs. 18 EPO of 53 in control females, 34%, $X^2 = 2.05$, $p=0.15$). Results to date suggest that T-females may be less active or attractive in mate choice; but conclusions will require that we complete the analysis of three more years of EPO data. Finally with respect to viability *T does not appear to influence survival* [annual return, T-females 32% (26/81), C-females 34% (32/95)], but data analysis is incomplete and we have one more annual census to conduct.

Figure 2 (left below). Mean response scores (±SE) extracted from a principal components analysis combining hits, dives, and nest checks performed by C- and T-females during the egg and nestling stages. A significant interaction indicates that control females stepped up defense when they had nestlings while T-females did not. Egg data from Clotfelter et al., 2004; nestling data from O'Neal et al. in prep. **Figure 3** (right, below). Nest survival rate varies by year, but T females have consistently lower nest survival than C-females (O'Neal et al. 200x).



Objective 2: To assess individual variation in hormonal responsiveness and to relate that variation to phenotype and fitness. *Proposed research will extend our knowledge of how testosterone relates to an array of phenotypic characters as well as to survival and reproductive success.*

Rationale.—There are, of course, limitations to the conclusions that can be drawn from implant studies (Zera et al. 2007). Implants can interfere with homeostatic mechanisms that normally regulate hormone secretion and with normal interactions among hormones. Hence a fuller understanding of the maintenance of variation in male and female testosterone requires that we assess **natural variation in T** and relate that variation to survival and reproductive success.

Progress to date, males.—Published results on juncos in VA reveal significant among-male variation in testosterone and testosterone-mediated characters. Specifically, males that readily elevate testosterone in response to physiological or social stimulation are more aggressive, more ornamented, and less parental. Conversely males that are less responsive to stimulation are less aggressive, less ornamented, and more parental. Further, males with higher initial levels of testosterone have less robust immune function (Table 1).

Trait	Initial T	Post T	Rise	+/-	Reference
Tail white	ns	ns	*	+	McGlothlin et al. 2008
Parental behavior	ns	< 0.1	*	-	McGlothlin et al. 2007
Aggressive behavior	< 0.1	*	ns	+	McGlothlin et al. 2007
Immune, IgG	*	ns	ns	-	Greives et al. 2006
Immune, complement	*	ns	ns	-	Greives et al. 2006

Table 1. Within-population co-variation in males between phenotype and T in response to GnRH: summary of published results. Traits: Tail white = sexually selected plumage trait (Hill et al. 1999, McGlothlin et al. 2005); parental behavior = feeding nestlings; aggressive behavior = response to a simulated territorial intrusion (STI); immune = IgG = circulating immunoglobulin G; complement = measure of innate immune function; *Initial* T = T prior to challenge with GnRH, *Post* T = T 30 min after GnRH challenge, and *Rise* = the difference between initial T and post T, +/- indicates nature of co-variation (positive or negative), * = $p < 0.05$, ns = not significant.

These results provide surprisingly strong validation for the conclusions drawn from the implant studies, which revealed that T enhances mating effort at the cost of parental effort and self maintenance. But the results also raise important new questions.

One **critical unanswered question** is the nature of the relationship between T in response to GnRH and survival and mating success. Results of our implant studies would predict directional selection on survival and fecundity, favoring males with lower values of T in response to GnRH, and directional selection on mating success, favoring individuals with higher values (Reed et al. 2006). Somewhat surprisingly, therefore, data obtained during two years (2003 and 2004, prior to the female implant studies) suggest that survival selection on T in response to GnRH is stabilizing and thus acting to maintain response to GnRH at intermediate values (McGlothlin et al. 200x)(Figure 4). Selection analyses of T in response to GnRH in relation to fecundity and mating success are underway (McGlothlin et al. in prep.)

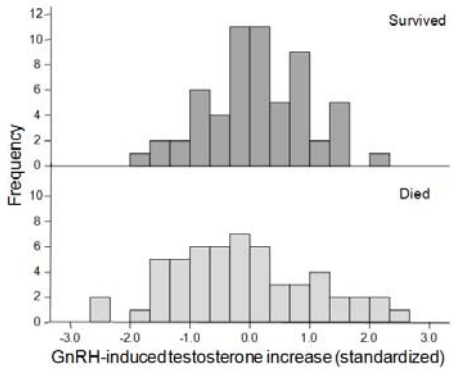


Figure 4. Frequency distributions represent GnRH-induced rise in T for individual males that survived and returned (top) to the following year or that failed to return (bottom). Variance was greater in non-survivors and selection was stabilizing (significance of selection gradients was tested using binomial logistic regression)(McGlothlin et al. 200x).

During 2007 we challenged 93 males with GnRH and monitored their nesting. We will challenge and monitor males again in 2008 and census in 2008 and 2009 to see whether the pattern uncovered by McGlothlin et al. (200x) persists or varies from year to year. The analyses will be done in collaboration with McGlothlin who is now a postdoctoral fellow at the U of Virginia.

Progress to date, females.—Like males, females vary individually in how much they elevate T in response to GnRH, but importantly they elevate T only when they are producing eggs (Jawor et al. 2007). Recently, we discovered co-variation between a female's tendency to elevate T and the concentration of T she deposits in her eggs, providing the potential to *assay an adult female's 'testosterone phenotype' by assaying her eggs* (Figure 5)(Jawor et al. 2007).

While many studies have addressed the effects of egg testosterone on offspring phenotype (e.g., Groothuis et al 2005; Groothuis and Schwabl 2008), relatively few have employed variation in egg

testosterone to predict phenotypic characters of females (but see Navara et al. 2006) or to relate those characters to female fecundity or fitness. Hence we propose to explore whether *yolk T as a proxy for T in response to GnRH* shows similar relationships to phenotypic characters as those previously observed in males.

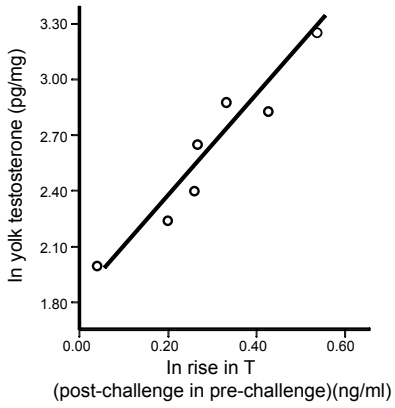


Fig. 5. Female rise in T in response to GnRH co-varies with yolk T. GnRH challenge was conducted during egg development or egg laying; T expressed as natural log (Jawor et al. 2007)

Using similar reasoning as that employed with males, we will ask whether females with higher levels of yolk T have whiter tails or suppressed immune function. We will employ field methods used in the earlier studies, i.e., census the population in early spring, collect blood samples for immunoglobulins (IgG)(Greives et al. 2006), measure tail white (McGlothlin et al. 2008). We will search for nests on a daily basis and when we find a nest prior to egg laying, we will mark the eggs each day as they are laid. When the clutch is complete, we will collect one egg (egg 3) and freeze it for later determination of steroid concentrations (Clotfelter et al. 2004). Nest predation is quite common, so the eggs we collect would rarely hatch even if not collected. Based on experience, we can expect to collect 30 eggs per season, which will be combined with ~75 eggs already collected. Analyses of these eggs will allow us to assess co-variation between yolk T and female tail white, season-long reproductive success, and annual return.

New studies will relate yolk T to female aggression, parental behavior, and fitness. Studies of aggression will be conducted using simulated territorial intrusions at the nest. We predict that females whose eggs have higher concentrations of T will be more aggressive toward an intruder. Using standardized protocols, we will flush the female from the nest, and while she is absent, place a caged female junco near the nest. We will measure the female's time to return, her closest approach including contact with the cage, aggressive vocalizations, and feather displays (Balph 1977). Our experience predicts that females will circle the cage and puff their feathers. Those that are more persistent or that produce more displays will be judged more aggressive. We will relate the female's behavioral response to yolk T in the collected egg. Using video analysis, at other nests we will quantify parental behavior (McGlothlin et al. 2007), predicting that if females resemble males, then females whose eggs have higher levels of yolk T will feed nestlings less often (McGlothlin et al. 2007). Finally, we will quantify fecundity and annual return (survival) in relation to yolk T.

By focusing on natural variation in females, these studies will complement results from implant studies and will allow us to determine the relative performance of females that produce eggs that are high or low in testosterone, thus contributing to our understanding of the co-evolution of hormonally mediated characters in males and females.

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

Rationale.— The goal is to determine whether patterns of natural variation in T observed within a population will generalize to other populations of the junco and in so doing ask whether the results provide support for phenotypic integration or independence. The working hypothesis under phenotypic integration is that hormone-phenotype relationships will be stable across populations and can be used to predict among-population variation in the levels of expression of T-mediated traits. Alternatively, under independence, hormone-phenotype relationships will vary among populations and trait expression will vary independently.

Prediction 1.—Under integration, the pattern of co-variation between T in response to GnRH and phenotype documented in VA predicts similar relations in other populations, i.e., males with stronger responses to GnRH will have whiter tails, be more aggressive and less parental. Under independence, T in relation to GnRH need not co-vary with phenotype as observed in VA. Study will focus on three additional populations: two in California [the mountain population residing in ancestral habitat (CA-A) and the urban population that recently colonized the UCSD campus (CA-C)] and the one that breeds in the Black Hills of South Dakota (SoDA)(see Table 2).

The CA populations are particularly interesting because the colonizing population entered the campus only very recently (~1980) and based on common garden results has undergone rapid evolution in the direction of lower tail white and smaller body size when compared to a nearby ancestral population (Yeh 2004). The CA populations also differ in aggressive and vocal behavior (Newman et al. 2006; Newman et al. 2008) and breeding phenology (Price et al. 2008). In fact, the complex of traits expressed in the CA-C population is just what would be predicted if the suite of character changes from the ancestral state were achieved by a dialing down of T in response to GnRH, as would be predicted by phenotypic integration. This leads to *Prediction 2*.

Prediction 2.—Within-population relationships between T in response to GnRH and tail white in VA may be used to predict that *among populations, rise in T should be greater* in populations with greater tail white and less in populations with less tail white. Similarly, aggression should be highest in the population with the most tail white (SoDA) and least in the population with the least tail white (CA-C). Conversely, based on the VA relationship between tail white and T in response to GnRH, parental behavior should be lowest in SoDA and highest in the CA-C)(see Table 2).

Progress to date.—In collaboration with T Price, we have studied the CA populations during 2006 and 2007; work in SoDA began in earnest in 2007. Thus far we have challenged more than 150 males with GnRH in CA and more than 80 males in SoDA. We have yet to determine whether T in response to GnRH co-varies with traits, as would be predicted from relationships in VA (Prediction 1). Information regarding Prediction 2 is presented in Table 2.

Table 2. Mean values for tail white, parental behavior and aggression across populations and in relation to T in response to GnRH. Data from VA are published; data for CA are currently under analysis by graduate student J Atwell; efforts in SoDA have just begun.

Trait	**VA**	**CA-A**	**CA-C**	**SoDA**
Tail white, sum	2.3[1]	2.64[7]	2.21[7]	3.57[9]
Tail white, %	40%	44%[7]	37%[7]	60%[9]
Parental behavior (visits/hr)	4.3[2]	2.53[8]	3.82[8]	-
Aggression (latency, songs)	115[3], 54[3]	-	-	-
T, rise spring (ng/ml)	4.2[4]	7.2[8]	6.1[8]	5.9[10]
T, post STI (ng/ml)	7.5[5]	6.0[8]	2.5[8]	-
Yolk T (pg/mg)	9[6]	-	-	-

[1-6]VA,Virginia, [1]Wolf et al. 2004, [2] Ketterson et al. 1992, [3] McGlothlin et al. 2007, [4]Jawor et al. 2006, [5]McGlothlin et al. 2008, [6]Clotfelter et al. 2004. [7-8]CA, California, A-ancestral, C-colonizing, [7]Price and Yeh in press, [8]Atwell et al. in prep., data still preliminary. [9-10]SoDA, South Dakota, [9]Ketterson and Nolan, unpubl., [10]Bergeon, Cain, et al. unpubl., data quite preliminary.

Tail white, sum = proportion of tail feathers that are white, summed over right side of tail; for CA we took published % times 6, then converted to a proportion. Tail white, %, = computed % of each feather that is white, summed over right side, divided by 6; for VA we took published sum of proportions, converted to a % , divided by 6.

Parental behavior = feeding nestlings; aggressive behavior = behavioral response to a simulated territorial intrusion; T, rise spring = increase in T after GnRH; T, post STI = T after STI; yolk T = egg yolk T.

The data (Table 2) from the two California populations support Prediction 2. T in response to GnRH and T after a simulated territorial intrusion (STI) are greater in CA-A than CA-C, as is tail white, while parental behavior is lower. Comparing SoDA to VA, T in response to GnRH and tail white are both higher in SoDA, as would be predicted. However, if we rank all 4 populations with respect to tail white, the match with T in response to GnRH is not complete. Additional data will provide greater confidence in any patterns, as some of these data are quite preliminary; but to date we see support for both integration and independence.

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

Rationale.—Hormonally mediated characters can differ in expression owing to differences in signal strength and target sensitivity, but the frequency with which these alterations arise independently or in tandem has received little study (but see, Silverin et al. 2004). Research conducted under this objective will compare individuals as strong or weak responders to GnRH according to how much they elevate T after a controlled dose of GnRH. We will ask first whether individuals are coordinated along the HPG axis, such that strong responders at the level of the pituitary are also strong responders at the level of the gonad (integration) or whether they are not (independence). We will then ask whether strong responders also have higher density of hormone receptors in key target tissues in the brain (integration), or whether, conversely, there is no relationship between hormone and receptor density (independence). On the assumption that within-population comparisons of strong and weak responders are revealing, future studies will compare males to females within populations, and males to males from closely related populations. The immediate objective is to integrate mechanistic and ultimate explanations for individual variation in mating effort and parental effort.

HPG axis, individual variation.—To repeat, juncos in VA vary in the degree to how much they elevate T in response to GrRh, and this repeatable variation correlates with behavior and morphology (Jawor et al. 2006; McGlothlin et al. 2007). However we do not know where along the HPG axis the individual variation resides, e.g. do individuals vary in the pituitary's response to GnRH (as measured by LH output in response to GnRH) or in the gonad's response to LH (as measured by T output in response to LH) or both? We will ask whether LH in response to GnRH correlates with T in response to LH. Under phenotypic integration, we predict a positive correlation; under phenotypic independence we predict no necessary association or even a negative correlation.

Methods.—We will capture 40 male juncos from the field in VA and transport them to Bloomington IN for study under controlled conditions. Each will receive a series of 3 challenges. The first challenge will measure T in response to GnRH, using already established methods, i.e., collect initial blood sample, inject intramuscularly with 1.25 μg of GnRH, and bleed again after 30 min (Jawor et al. 2006). The second challenge 5 days later will be either with GnRH or with LH. The third challenge, 5 days after the second, will be GnRH or LH, whichever was *not* injected in the second challenge, thus controlling for order. For the second GnRH challenge, the dose of GnRH will not vary from established methods, but the bleed for LH will come sooner because this hormone will peak sooner and we wish to capture maximum response. We will conduct pilot testing for the best time by bleeding a series of juncos (not from this experiment) at 5 min intervals to determine when the maximum level is achieved. The dose of LH that is needed to achieve maximum levels of T is an empirical question and will also need to be determined in pilot tests. LH levels 30 min after GnRH are known to be 2-3 ng/ml (Jawor et al. 2006). Estimates from the literature (Hector et al. 1990) or known plasma concentrations of LH in the junco indicate an intramuscular dose of ~ 0.5 μg LH/50 ul of buffered saline, and we will titrate around this

dose. The T assays will be run in our laboratory; George Bentley has agreed to run the LH assays (U California, Berkeley, please see letter of support).

We will quantify co-variation between the LH response to GnRH and the T response to LH using correlation analysis. We will also ask which measure is the better predictor of T in response to GnRH as measured in the first challenge. If the responses of pituitary and gonad to the second and third challenges are positively correlated, *we will interpret this to mean that individuals tend to be strong or weak responders along the HPG axis,* as would be predicted by phenotypic integration. No relationship will indicate independence.

Variation in target sensitivity to testosterone.—We will compare neural sensitivity of strong and weak responders to GnRH using two techniques, immunocytochemistry (ICC), which allows visualization and quantification of cells containing receptor proteins, and *in situ* hybridization, which allows visualization and quantification of mRNA. The ICC work will be done in collaboration with Dale Sengelaub (Indiana U, please see appendix for letter of support) and will focus on androgen receptor (AR); the *in situ* hybridization will be done in collaboration with Barney Schlinger (UCLA, please see appendix for letter of support) and will focus on mRNA for androgen receptor (AR), estrogen receptor (ER_α) and aromatase (AROM), the enzyme that converts testosterone to estradiol.

The study will employ the same 40 juncos just described for measuring sensitivity along the HPG axis. After the initial GnRH challenge, we will immediately run EIAs to assess T in response to GnRH to identify the strongest and the weakest responders. We will select the top and bottom 12 responders to increase the likelihood of showing that strong and weak responders do or do not differ in their target sensitivity. Five days after the third challenge described above, we will perfuse and collect tissue. Six of the 12 from each category will be used for ICC; 6 for *in situ* hybridization.

ICC methods.—Prior to sacrifice, males will be injected (pectoral muscle) with T in 0.1 ml peanut oil (10 mg/kg) to enhance AR staining. Ninety minutes later, birds will be anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains and gonads will be removed and post-fixed briefly in the same fixative, and cryoprotected overnight in 30% sucrose phosphate buffer. Frozen sections will be cut coronally at 30 μm on a sliding microtome, collected into alternate series, and processed free-floating. After blocking of endogenous peroxidase activity and nonspecific secondary antibody binding, sections will be incubated with anti-AR (Upstate, PG-21 directed against the first 21 amino acids of the N-terminus of the human AR protein, 1:100)(compare Soma et al. 1999) for 1 hour at room temperature; control sections incubated without the primary antibody or after antibody preabsorption will also be generated. After rinsing with PBS, sections will be incubated in biotinylated goat anti-rabbit (Santa Cruz, 1:500), rinsed again with PBS, and then incubated with horseradish-peroxidase conjugated streptavidin (1:500). Staining will be visualized using DAB, and sections will be dehydrated in ethanol, cleared in xylene, and cover slipped. A separate series of alternate sections will be stained with thionin for use as a reference set. Using a computer-based image analysis system interfaced with a microscope (Stereo Investigator, MicroBrightField, Inc.), the volumes of the relevant brain nuclei will be measured and the density of AR+ cells determined using random systematic sampling.

In situ hybridization methods.—Detailed methods appear in Canoine et al. (2007) and are summarized here briefly. Similar to the ICC, we will deeply anaesthetize using an overdose of isoflurane, perfuse with 30 mL 0.9% saline, followed by ice-cold 4% neutral buffered formaldehyde (Sigma) via peristaltic pump. We will dissect brain, gonad and adrenals, which we will postfix for 2 h in 4% formalin, cryoprotect by immersion in 20% sucrose in phosphate buffer (PB), then in 30% sucrose in PB (at 48C overnight), freeze on dry ice, store at -80^{o}C, and section later (at 16 μm) using a cryostat into 5 series through the whole brain. The protocol for the hybridization will employ a probe for androgen receptor (AR) that was

developed from canary; probes for ER and AROM were developed from zebra finch. Hybridization intensity will be quantified using image analysis (Scion) and measured as optical density on films and silver grain density on emulsion-dipped slides. Analysis will be based on pair-wise comparisons of 6 dyads of bird randomly selected from the 12 strong and 12 weak responders and processed for mRNA in the same run. *Targets to be studied* will be those associated with aggressive behavior (nucleus taeniae, nT), song (e.g. HVC), reproductive behavior (e.g. the pre-optic area of the hypothalamus, POM), and negative feedback in the regulation of the HPG axis.

Interpretation.—Co-variation between signal (T in response to GnRH) and target (number of target cells and hybridization intensity of receptor/enzyme) will be taken as evidence for the existence of strong and weak responders 'system-wide,' in that individuals producing higher levels of T in response to GnRH would also be transcribing higher quantities of the mRNA for AR, $ER_{\alpha,}$ or AROM in target tissues, or would have greater numbers of cells translating AR in these same tissues. In regions regulating the HPG axis we predict that strong responders to GnRH will have fewer AR+ cells in regions related to regulation of the HPG axis (negative feedback). In this form of phenotypic integration, signal and target sensitivity might evolve together, as selection for one could lead to selection for the other. Lack of correspondence in signal and target sensitivity will be taken as evidence of phenotypic independence. Caveats about plasticity and context dependence will clearly need to be considered, but to our knowledge this will be one of the first attempts to assess co-variation of signal and target sensitivity using these techniques.

Summary.—Research described in this proposal will compare resemblance between the sexes in their phenotypic sensitivity to experimentally elevated testosterone, which in turn will predict the potential for both direct and correlated responses to selection (Fig. 1). If the sensitive traits are beneficial in one sex and detrimental in the other, they will provide evidence of constraint. Research will also reveal how natural variation in T relates to phenotype and fitness in both sexes within a population. Across populations, it will reveal whether hormonal traits are tightly integrated in their expression or more independent. Finally, the research will assess the linkage between signal and target, again to determine relative integration and independence. The junco provides an unusual opportunity to understand how evolution interacts with hormones to give rise to differences and similarities between the sexes, among individuals, and across populations.

Broader impacts: Enhancement of education/outreach/societal implications.—Research proposed here will provide opportunities to train future scientists, some of whom will be recruited from Indiana University's REU program in Animal Behavior, which is focused on members of groups underrepresented in science http://www.indiana.edu/~animal/academics/reu.html. It will also enhance the quality of three university classes taught by Ketterson: an undergraduate class in Biology of Birds and two graduate classes, Behavioral Ecology and Professional Ethics for the Bio-behavioral Sciences. Past NSF support has allowed me to bring personal experience to classroom, field and lab exercises, and discussions of ethical issues. The research has also been a training ground for field techniques and the integration of proximate and ultimate approaches to behavior and physiology as evidenced by the many participants who have gone on to careers in science. Graduate students supported by the research judge science fairs, give presentations to the public, and disseminate findings to the media. Potential societal implications of the research include implications for (1) impact of endocrine disrupting chemicals in the environment, (2) a greater understanding of the relationship between sex and gender, and (3) population-level responses to environmental change.