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).

PROJECT SUMMARY

Males and females typically share some attributes and differ in others. Differences arise when one sex is subject to greater reproductive competition than the other (*sexual selection*) and when the two sexes differ in their ecology (*natural selection*). Because the sexes share most of their genome, they are also shaped by *correlated responses to selection*, which can hasten evolution if attributes directly favored in one sex also benefit the other, or serve as a constraint if an attribute favored in one sex is disadvantageous to the other. Over time, one sex may escape constraints imposed by the other through the evolution of independent mechanisms of trait expression (*sexual independence*).

Proximate mechanisms underlying similarities and differences between the sexes include sex chromosomes, cell-autonomous gene expression, and circulating hormones that mediate the expression of suites of coordinated traits (hormonal pleiotropy). Gonadal steroids can act early in development to organize tissues that are, or are not, activated by a steroid later in life (hormone-mediated sex-limited expression). Gonadal steroids can also regulate gene and trait expression in adults of both sexes, and, when they do, such traits and the hormones that mediate them are subject to direct (natural and sexual) selection within each sex, as well as correlated responses to selection across sexes.

The focus of this proposal is the steroid hormone testosterone and its integrating effect on sex differences and resemblances in adult males and females of a songbird species, the dark-eyed junco. The research has four objectives:

1) To experimentally elevate testosterone (T) in females, measure its phenotypic effects, and compare them to previous results from males. New experiments will address T's effect on female attractiveness and parental behavior towards nestlings. Studies of hand-raised offspring will reveal whether experimentally elevated maternal T influences offspring plumage.
2) To determine fitness consequences of experimentally elevated T in free-living females. Proposed studies will provide evidence of adaptive or maladaptive outcomes of elevated T in females.
3) To assess individual variation in hormonal responsiveness and the potential for correlated response to selection. Planned studies will show whether response to GnRH co-varies with phenotype or fitness. Greater response in individuals with more mates or greater fecundity would suggest a role for T in sexual selection; study of relatives will indicate potential for correlated responses.
4) To assess sexual independence by comparing T's role in the activation of aggressive behavior. Planned experiments will assess whether repeated intrusions cause residents females to elevate T in response to an intruder or whether other hormones mediate female aggression and competition.

Intellectual merit.—Research described here will make important contributions to our knowledge of sex differences: how they are regulated and how they evolve. By focusing on the phenotypic effects of one integrating, signaling molecule, testosterone, and one system that has received intensive, long-term study, the work will address the interacting effects of natural, sexual and correlated responses to selection in order to better understand adaptation and constraint.

Broader impacts.—Research described here will provide opportunities for training future scientists of diverse backgrounds in the conduct of laboratory and field experiments that address the integration of proximate and ultimate explanations for sex and gender differences. It will also enrich graduate and undergraduate courses taught at Indiana University. Potential societal implications include (1) relevance to the study of hormonally active agents in the environment, (2) improved methods for breeding captive songbirds for conservation, and (3) a greater understanding of the relationship between sex and gender.

PROJECT DESCRIPTION

RESULTS FROM PRIOR NSF SUPPORT

NSF/IBN-02-16091 to Ellen D Ketterson, Indiana University, Bloomington, $447,792 (including $6,000 REU supplement), 07/31/02 – 07/31/05, "Sex differences and similarities: natural, sexual, and correlated responses to selection"

Since1986 our group has studied the steroid hormone testosterone and its integrating effect on behavior and physiology in a songbird species, the dark-eyed junco. Earlier proposals focused on males, while this award focused on females. Listed here are the research objectives as they appeared in the original proposal, with progress indicated in parentheses.

(1) to assess the role of testosterone in sexual integration by comparing natural male and female hormone profiles, hormonal response of females to stimuli known to increase T in males, and hormone levels in females that do and do not produce young by extra-pair fertilizations (EPFs). (Jawor et al. submitted, Ketterson et al. in press, data analysis underway, work will continue).
(2) to alter the level of circulating testosterone experimentally and compare effects of altered testosterone in females to previous results from males. (Clotfelter et al. 2004, McGlothlin et al. 2004, Ketterson et al. in press, Zysling et al. in prep., work will continue).
(3) to determine whether female responses to altered testosterone are beneficial, neutral, or detrimental in the field. (Ketterson et al. in press, data analysis underway, work will continue).
(4) to compare how degree of relatedness affects a sexually selected trait and two hormone-related traits. Juncos of known genetic relatedness will be assessed for family resemblance in measures of body size and a plumage trait that affects attractiveness. Additional juncos will be reared and compared for three hormonally mediated traits. (Wolf et al. 2004, McGlothlin et al. in press, data analysis underway, work will continue).

Contribution to the development of human resources.—Numbers of students receiving training with the help of IBN-02-16091: 6 post-doctoral associates (1 male, 5 female), 7 graduate students (1 male, 6 female, 1 African-American), 14 undergraduate or post-undergraduate students (4 male, 10 female, 2 African-American, 1 Hispanic, 1 first-generation)(8 also supported by REUs, 2 by Medic-B, 5 have entered graduate school, and 7 are co-authors on submitted manuscripts or publications).
Post-doctoral associates: **J Casto** (now Research Director, CINF/Adjunct Illinois State U), **E Clotfelter** (now Asst. Prof., Amherst College), **D Duffy** (still in training, SiT, U of Pennsylvania), **J Jawor** (SiT), **M Sandell** (now researcher, Lund U, Sweden), **W Reed** (now Asst. Prof., North Dakota State U)
Graduate students: **N Gerlach** (SiT), **J Grindstaff** (now post-doc Lund U, SW), **B Heidinger** (SiT)**, J McGlothlin** (SiT)**, D O'Neal (SiT), S Schrock** (SiT), **B VanRoo** (now Asst. Prof. Framingham State U), **D Zysling** (SiT)

Publications resulting from the current award, 2002-04.—Total is 19. Included in total are 9 articles in manuscript form, of which 2 are under review, 6 are under revision after submission, and 1 is about to be submitted. Also included are 2 manuscripts that have been accepted, and 8 already published articles. Listed here are the 11 most directly related to objectives 02-16091.

Zysling, D.A., Greives*, T.M., Breuner, C., Casto, J.M., Demas, G.E., and E. D. Ketterson. 200x. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis*): implications for the evolution of sexual dimorphism. *Proceedings Royal Society, B*, soon to be submitted.

Jawor, J, ,Young*, R., and E.D. Ketterson. 200x. Females competing to reproduce: dominance matters, testosterone may not. *Hormones and Behavior*, submitted.

Reed, W.L, Clark, M.E., Parker, P.G., Raouf, S.A., Arguedas, N., Monk, D.S, Snajdr, E., Nolan, V. Jr., and E.D. Ketterson, E.D. 200x. Physiological effects on demography: A long-term experimental study of testosterone's effects on fitness. *American Naturalist,* submitted, under revision.

Duffy, D.L, McGlothlin, J.W., and E.D. Ketterson. 200x. Does social status influence mate preference in dark-eyed juncos (*Junco hyemalis)*? *Animal Behaviour*, submitted, under revision.

Ketterson, E.D., V. Nolan Jr., and M. Sandell. 2005. Testosterone in females: implications for the evolution of sexual dimorphism. *American Naturalist,* invited ms, submitted.

McGlothlin, J.W., Parker, P.G., Nolan, V. Jr., and E.D. Ketterson 2005. Correlational selection leads to genetic integration of body size and an attractive plumage trait in dark-eyed juncos. *Evolution,* in press.

McGlothlin J.W.*, Neudorf, D.L.H., Nolan, V. Jr., and E.D. Ketterson. 2004**.** Elevated testosterone reduces choosiness in female dark-eyed juncos (*Junco hyemalis*): evidence for a hormonal constraint on sexual selection? *Proc. R. Soc. Lond B* 271:1377-1384.

Clotfelter E.D., O'Neal*, D.M., Gaudioso*, J.M, Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L., Nolan, V. Jr., and E.D. Ketterson. 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? *Hormones and Behavior* 46:171-178.

Wolf, W*, Casto, J.M., Nolan, V. Jr., and E. D. Ketterson. 2004. The effect of female ornamentation on mate choice by male dark-eyed juncos. *Animal Behaviour* 67 (1): 93-102.

D.L. Neudorf, Ziolkowski, D.J., Jr, V. Nolan Jr., and E. D. Ketterson. 2002. Movement by female dark-eyed juncos during the fertile period suggests that males visit females for extra-pair copulations. *Ethology* 108:1-15.

**Indicates authorship by an undergraduate student*

MAJOR OBJECTIVES OF NEW RESEARCH

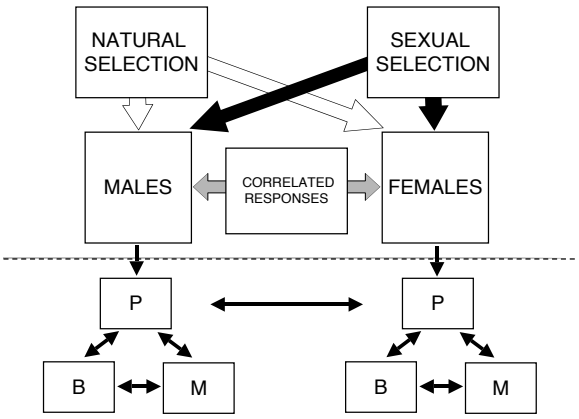
This proposal addresses adaptation and constraint by focusing on ultimate and proximate bases of sex differences in a weakly dimorphic songbird, the dark-eyed junco. We propose to manipulate levels of testosterone in females to reveal the degree of hormonal correlation between the sexes and also to assess costs and benefits so as to simulate natural and sexual selection. We will also document natural variation in testosterone in response to a hormonal stimulus, gonadotropin releasing hormone (GnRH), in order to assess the potential for correlated responses to selection. Finally, we will explore the potential for sexual independence by comparing how females mediate aggressive behavior with hormones. Results will help to resolve important issues in the regulation and evolution of sexual dimorphism.

INTRODUCTION

Biologists have long recognized that traits that enhance fitness in males are not necessarily the same as those that enhance fitness in females (Andersson 1994; Darwin 1871). Males typically invest more time and energy in attracting or pursuing mates; females invest more in caring for offspring. Among males, competition for mates (*sexual selection*) often leads to a few highly successful males that sire many offspring, and other males that sire no offspring at all; reproductive success in females typically varies less (Shuster and Wade 2003). As a consequence, traits that enhance mating success may be expressed only, or more strongly, in males than in females, leading to sex differences.

Natural selection can also generate differences between the sexes. If males and females differ in their ecology, natural selection will favor attributes that suit each sex to its environment, and these attributes may differ (Karubian and Swaddle 2001; Ligon 1999; Selander 1966). Conversely, if the sexes are quite similar in their ecology, to the extent that they compete with one another, natural selection may lead to character displacement, causing the sexes to diverge (Johnson and Macdonald 2001).

In addition, the evolution of sex differences may be influenced by *correlated responses to selection*.



If a trait that would be advantageous to one sex is detrimental to the other sex, evolution of the trait may be constrained, and the sexes may be more similar than would be 'optimal' for either considered alone (Lande 1980; Lande 1987; Lande and Arnold 1983; Price and Burley 1994). Correlated responses occur because the sexes share almost all of their genomes, and shared loci may influence trait expression in both sexes. The ensuing selection on males and females may be antagonistic or reinforcing (Chippindale et al. 2001; Rice 1996; Rice and Chippindale 2001) and thus check or hasten evolution. The balance achieved between sex differences and resemblances is a consequence of a network of selective forces and responses to selection, some of which act independently on each sex and some of which act in concert (as summarized in Fig. 1).

Of the multiple interactions among natural, sexual, and correlated responses to selection in males and females depicted in Fig. 1, the least studied are sexual selection on females and correlated responses in both sexes (Amundsen 2000; Jones and Hunter 1999; Potti and Merino 1996). Progress in understanding the evolution of sexual dimorphism will require not only that more attention be paid to these processes but also more knowledge about how they interface with the proximate mechanisms that give rise to sex differences.

Mechanistically and developmentally, the sexes are joined and differentiated by multiple mechanisms, e.g., maternal imprinting, sex-linked genes, the action of steroidal and non-steroidal hormones (Nelson 2000). Also important are sex differences at the cellular level that are independent of hormones (Arnold and Schlinger 1993; Arnold 1996; Arnold 2004; Wade et al. 1999), as well as various forms of ontogenetic integration (Badyaev and Hill 2000; Badyaev et al. 2000). Among these mechanisms is an important mediator of sex differences and resemblances, the steroid hormone testosterone (T).

Testosterone's multiple effects on the morphological (M), behavioral (B) and physiological (P) phenotype are depicted below the dotted line across Figure 1. These effects have been well characterized in males (Ketterson et al. 1999), as has the action of natural and sexual selection on testosterone-mediated traits in that sex (Klein et al. 1997; Ketterson et al. 1999; Wingfield et al. 2001; Westneat et al. 2003; Reed et al. 200x). Females also produce testosterone, but its effects on the female phenotype have been less well documented (but see Staub and De Beer 1997; Van Duyse et al. 2002; Ketterson et al. 200x). Consequently, we rarely know which aspects of the female hormonal phenotype might be the result of direct (natural and sexual) selection, and which might

represent correlated responses to selection on males, and which may be both. Neither do we know how often existing male phenotypes might represent compromises between the advantages of testosterone-mediated characters in males and costs of such characters in females. Research described in this proposal is designed to help answer these questions.

RESEARCH OBJECTIVES AND PREDICTIONS

1) To experimentally elevate testosterone (T) in females, measure the phenotypic effects, and compare them to previous results from males. The extent of resemblance between the sexes in their phenotypic sensitivity to implants will predict the potential for direct and correlated responses to selection. Traits that are sensitive to an increase in T in only one sex should not be subject to correlated responses in phenotype; traits that are sensitive in both sexes may respond directly or in correlated fashion. Completed experiments on both sexes have shown similar effects of experimentally elevated T on aggression, levels of other hormones, and immune function; whereas a trait sensitive that is sensitive to T in males, nest defense behavior, was not sensitive in females. New experiments will address T's effect on female attractiveness and parental behavior towards nestlings. Studies of hand-raised offspring will reveal whether experimentally elevated maternal T influences offspring plumage.

2) **To determine fitness consequences of experimentally elevated T in free-living females**. Traits that are sensitive to elevated T in both sexes, but that are beneficial in one and detrimental in the other, provide evidence of constraint. Alternatively, altered traits that have fitness consequences in only one sex, leave the sexes freer to evolve independently. Completed experiments have shown higher fitness in T-treated males than in controls, suggesting higher T would benefit males. Proposed studies will provide evidence of adaptive or maladaptive outcomes of elevated T in females.

3) **To assess individual variation in hormonal responsiveness and the potential for correlated response to selection**. Natural variation in T is clearly critical to predicting co-evolution of the sexes. If the sexes are hormonally correlated, they are inter-dependent; if they are not, they are freer to evolve independently. Studies underway have shown that male juncos and laying females vary in how much they elevate T in response to a standardized injection of gonadotropin releasing hormone (GnRH). Planned studies will show whether response to GnRH co-varies with phenotype or fitness. Greater response in participants in individuals with more mates or greater fecundity would suggest a role for T in sexual selection. Additional study of relatives will indicate potential for correlated responses to selection.

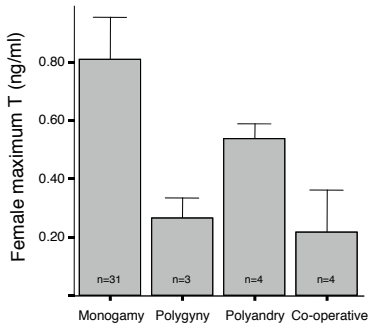
4) **To assess sexual independence by comparing T's role in the activation of aggressive behavior**. Sex differences in the hormonal mediation of the same trait predict sexual independence; similarities in mediation predict correlated responses and the potential for constraint. Completed studies of aggressive behavior have shown that experimental elevation of T enhances the aggressive responses of resident females to an intruder, but intrusions do not cause residents to elevate endogenous T. Planned experiments will assess whether repeated intrusions cause residents females to elevate T, or whether other hormones are involved in the mediation of female aggression.

RELATION TO PRESENT STATE OF KNOWLEDGE IN THE FIELD

Testosterone.—Testosterone, often regarded as the 'male hormone' because of its role in sex-limited trait expression (Nelson 2000), is secreted primarily by the gonad, but also by other tissues, e.g., the

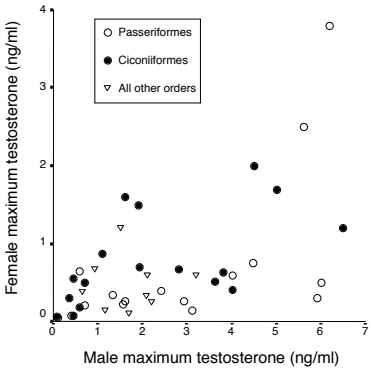
adrenal gland in birds and mammals (Arnold 2004; Freking et al. 2000). Once secreted, T enters the circulation, from which it may bind with androgen receptors and join with co-activators to initiate gene transcription or, as one alternative, be converted to other steroids that interact with other receptors, co-activators and genes. These receptors, co-activators, and conversions to and interactions with still other hormones allow T to mediate multiple and varied phenotypic effects and to exemplify hormonal pleiotropy and epistasis as described by Finch and Rose (1995; see also Ketterson and Nolan 1999). Many traits are organized by early developmental exposure to steroids and later activated by T. Recognizing the importance of all these interactions, this proposal focuses on the activating effect of plasma T on the phenotype of adults.

T in females.— The primary sources of circulating T in female vertebrates are the ovary and the adrenal (Nelson 2000). Female androgens have been implicated in many different functions (for review see Staub and De Beer 1997). In some cases target tissues in males and females are the same, while in others, target tissues are specific to females, e.g. T's impact on the ovary (Johnson 2000). Plasma levels of T in female vertebrates have been shown to vary developmentally and, in adults, annually, seasonally, and with stage of reproduction (Callard et al. 1978; Staub and De Beer 1997; Ketterson et al. 200x). In birds, females exhibit maximum values of T at the beginning of the breeding season just prior to and during the time they lay eggs (Ketterson et al. 200x).



Does T co-vary with traits that benefit females?— Wingfield et al. (1994; 2000) report greater sex differences in T in species with greater sexual dimorphism. Ketterson et al. (200x) have also shown that maximum seasonal values of T vary among female birds in relation to mating system. Females of socially monogamous species, which might be expected to compete for mates and parental care, have the highest levels of T as compared to females from other social systems (polygyny, polyandry, and cooperative breeding) (Ketterson et al. 200x)(Fig. 2), suggesting a role for T in female-female competition.

Do male and female T co-vary?—If female T is the result of correlated responses to selection on male T, we would predict co-variation across species in male and female levels of T, and there is evidence for this as well. Fig. 3 plots published, maximum values of T across species and shows co-variation between the sexes. Visual comparison of the sexes in Fig. 3 indicates species in which T co-varies in males and females and others in which female T is low, regardless of the level of male T. When treated as statistically independent, the data reveal a novel result: female levels are significantly correlated with male levels overall, and within two separate orders, the Ciconiiformes and Passeriformes (Ketterson et al. 200x). Summarizing the last two paragraphs, phylogenetic analyses will require more data than are currently available, but at present there is evidence both for co-variation between the sexes and for independence.



Individual variation.— The prevailing view among behavioral endocrinologists is that circulating levels of hormones are not related linearly to phenotypic expression (Adkins-Regan 2004, in press; Frank 1997; Hews and Moore 1997). Instead, traits are expressed when a threshold level is exceeded. Nevertheless, a number of studies have demonstrated

co-variation between plasma levels of T and phenotypic expression of T-mediated traits. Examples from males include jungle fowl (comb length)(Zuk et al. 1995), barn swallows (vocalizations) (Galeotti et al. 1997), and bighorn sheep (aggressiveness) (Pelletier et al. 2003; Silverin et al. 2004). Examples from females include mountain spiny lizards (aggressive behavior) (Woodley and Moore 1999), European Starlings (immune function)(Duffy et al. 2000), and dunnocks (vocalizations) (Engelhardt von et al. 2000; Langmore et al. 2002). Research described in this proposal will clarify the relationship between T and phenotype for both sexes in a set of fitness-related behaviors.

Heritability and genetic correlations.—Most of the available data on the inheritance of T-levels or T-mediated characters come from agricultural animals or humans, not from natural populations, and especially not from females (summarized in, Ketterson et al. 200x). Response to a challenge with gonadotropin releasing hormone (GnRH) is significantly heritable in boars (Robison et al. 1994) and beef cattle (Davis 1993), and testis size, a character that correlates with testosterone, is heritable in sheep (Matos and Thomas 1992). In the side-blotched lizard, throat color, which is related to testosterone and progesterone is heritable in both males and females and is genetically correlated across the sexes (Sinervo and Zamudio 2001).

Correlated responses to selection.— Several studies, again in agricultural and laboratory species, report correlated responses in females to selection on hormone levels or hormone-mediated characters in males, as would be predicted if the sexes sometimes constrain one another's evolution (Robison et al. 1994;McNeilly et al. 1988; Haley et al. 1989). In mice, for example, selection on male aggression led to correlated responses in maternal and predatory aggression in females (Sandnabba 1996), and in a natural population of lizards, cyclical episodes of selection on throat color produced correlated responses in males and females (Sinervo and Zamudio 2001).

Hormonal basis of female aggression.—The endocrine system also provides potential means of escape from constraints imposed by correlated responses to selection. Traits that are common to both sexes but are differently employed may come to depend on different hormonal mechanisms to regulate their expression, and when that occurs the trait becomes free to evolve independently. Female aggression, for example, may or may not be a trait that is regulated differently in male and female birds. Experimental elevation of T has often been shown to enhance aggressive behavior and social status in males (Balthazart 1983; Dufty and Wingfield 1986). Females, although studied less, are also known to become more aggressive in response to experimentally enhanced T (Searcy 1982; Adkins- Regan 1999;Zysling et al. 2003). With respect to a social 'challenge,' T rises in females of some species after a simulated intrusion (Langmore et al. 2002), but not in others (Elekonich 2000; Hau et al. 2004), an inconsistency that will be addressed by this proposal. Females may depend on different hormones to mediate aggression; likely candidates are Progesterone (P), estradiol (E_2) and dehydroepiandrosterone (DHEA) (Hau et al. 2004; Soma et al. 2001; Soma et al. 2002).

To summarize, the comparative literature shows natural variation in female T and thus the potential for direct selection on T-mediated traits as well as correlated across-sex responses to selection. Trait values sometimes co-vary with T-levels, and traits and levels are heritable both within and across sexes. Single traits may rely on different mechanisms in males and females. All told, however, conclusions are quite tentative, because data on females and natural populations are rarely available.

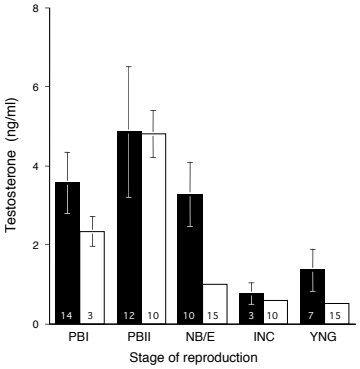
BASIC METHODS AND PRELIMINARY DATA

Study species.—The dark-eyed junco is a well studied songbird (e.g., Deviche et al. 2001; Deviche et al. 2000; Nolan et al. 2002) that is 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; McGlothlin et al. in press; Wolf et al. 2004). The sexes differ qualitatively in vocal behavior (only males typically sing) and parental behavior (only females incubate). Juncos are abundant throughout North America and *J. h. carolinensis* breeds in the Appalachian Mountains. Carolina juncos are territorial when breeding and form socially monogamous bonds, but frequently produce young via extra-pair fertilizations (~35%)(Raouf et al. 1997; Ketterson et al. 1998; Reed et al. 200x). Females build the nest and incubate eggs; both sexes care for nestlings and fledglings. Pairs can produce 2-3 broods per season, but rarely do because nest predation is common in most years. Fidelity to previous year's breeding sites is nearly complete among males, less so among females, and least among offspring (~15%). Each year the study area supports individuals of varying degrees of relatedness (e.g. grandparents, parents, sibs, half-sibs, etc.) (McGlothlin et al. in press).

Study sites.—When studying juncos in the wild, we work at Mountain Lake Biological Station (University of Virginia), near Pembroke VA. When studying captive juncos, we house them either at the biological station in Virginia or in Indiana at a temperature- and day-length-regulated 11-room indoor aviary or an outdoor aviary that can be divided into 30 breeding compartments or serve as a single enclosed space in which juncos can live semi-naturally. Juncos thrive in captivity as evidenced by high survivorship and excellent physical condition. When held indoors they readily initiate reproduction and exhibit normal behavior during courtship, nest building, egg laying and incubation, but are not yet reliable at rearing nestlings.

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), and, for some experiments, we use an EIA kit (Assay Designs, Inc., #901-065) to measure T (Clotfelter et al. 2004). The EIA kit generates higher values of T than the traditional RIA and is more expensive, but it equals the RIA in terms of inter- and intra-assay variation. Further, it is more sensitive, can be conducted in far less time, and, most importantly, requires smaller volumes of plasma, which is more appropriate for the repeated sampling that is part of the GnRH challenges we propose below. For DHEA, we have used the tritium-based RIA, but following the advice of K.K. Soma, we propose to measure DHEA using a kit (Diagnostic Systems Laboratories, DSL, catalog # 8900) that is highly sensitive (down to 20 pg/ml) and effective with small volumes of plasma. (50-75 ul). We will follow Granger's (1999) modification of the kit's instructions as advised by Soma (pers. comm.).



Sex differences in plasma T vary seasonally in juncos and levels in females can be high.— A seasonal profile of T in free-living male and female juncos shows that the sexes resemble one another (see Fig. 4), males in black, females clear). Based on 101 blood samples collected during one breeding season and classified according to 5 stages of reproduction, (1) pre-breeding I (< 1April), (2) pre-breeding II (> 1 April, individuals not known to be breeding) (3) females building nests or laying eggs and males presumably mate-guarding, (4) females incubating, and (5) males and females tending nestlings, T was highest during stage (2), i.e., prior to breeding in both sexes. Later it declined, faster in females than in males, and rebounded slightly during the nestling stage in males only (Ketterson et al. 200x).

Manipulating T in females.— To prolong the natural early-season peak values of T in females, we place silastic implants (1.5 mm i.d., 2.0 mm o.d.) subcutaneously along the flank, but employ smaller doses than previously used with males (one 5mm implant, as opposed to two 10mm implants in males). Controls receive empty implants. We assign treatment at random, block by age and, where relevant, keep treatment constant in females that return in successive years. After implanting, we recapture females with minimal disturbance, verify that implants are in place, and obtain plasma samples. Implants are removed at the end of the breeding season. We have assessed the impact of implants on plasma T in four sets of experiments (Clotfelter et al. 2004; McGlothlin et al. 2004; Parker-Renga et al. 200x; Zysling et al. 200x) and know that the values induced closely resemble the early season, natural female peak. Implants do not affect circulating levels of DHEA or estradiol (unpublished data), but they do elevate CORT and CORT binding globulin (Clotfelter et al. 2004; McGlothlin et al. 2004; Parker-Renga et al. 200x; Zysling et al. 200x). Importantly, T-implants significantly increase yolk levels of T in the eggs laid by T-implanted females (Clotfelter et al. 2004).

Other methods.—Our group has successfully used all the methods proposed for the experiments described below. These include bleeding nestlings and adults for hormones for DNA, performing steroid RIAs on plasma (Schoech et al. 1998) and egg yolks (Casto et al. 1999; Lipar and Ketterson 1998) and EIAs on plasma (Clotfelter et al. 2004), video taping at nests (two 90- min video taped sessions/nest, one in AM, one in PM)(Clotfelter et al. 2004), quantifying variation in melanin-based plumage coloration (Wolf et al. 2004b), assessing attractiveness in mate choice trials (Enstrom et al. 1997; Hill et al. 1999; McGlothlin et al. 2004), and monitoring nests and egg-nestling condition (egg size/body mass/linear dimensions), as well as documenting nest success, year-to-year site fidelity of young and adults, mate fidelity, and survivorship (Reed et al. 200x). We have also hand-reared offspring using a diet consisting of boiled egg, ground carrot, and commercial dried dog food, supplemented with vitamins and bee moth larvae (Ketterson et al. 1992). These young were released and returned to breed successfully in subsequent years.

Since 1990, we have routinely collected blood samples for paternity analysis (first mini-satellites, more recently microsatellites, past work performed in collaboration with P. G. Parker). To determine parentage, we extract DNA with phenol/chloroform, precipitate with ETOH, and amplify by PCR using a Qiagen multiplexing kit and a protocol developed for juncos by Trevor Price, U of Chicago. We use an ABI3730 sequencer and Genemapper software to read fragments generated by PCR. Samples are genotyped two times andd also hand-read; we use a statistical program to identify clusters of fragments as alleles. We have developed and characterized 7 variable loci in juncos [Dpu01 (14 alleles), Dpu16 (13 alleles), GF01b (14 alleles), GF05 (13 alleles), GF06 (5 alleles), GF14 (14 alleles), and Hru5 (8 alleles)] and discontinued use of Mau 23 that shows no variability.

To summarize, we already know that males and females resemble one another naturally in their testosterone profiles except when females are building nests and laying eggs and males are presumably mate guarding. Manipulations of T induce levels in females seen naturally in early spring. Females are not harmed by implants, and the implants do not elevate circulating levels of DHEA or estradiol, although they do elevate corticosterone. Other necessary methods have been developed, tested, and are known to work.

PROPOSED STUDIES

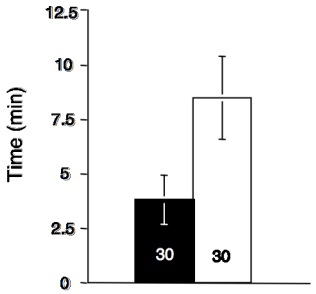
Objective #1: To experimentally elevate testosterone (T) in females and compare the phenotypic consequences to previous results from males.

Rationale.— Rather than select artificially to monitor correlated responses in males and females (Delph 1999) or use selection experiments to detect possible intersexual conflict (Rice 1996), we propose *to simulate selection experimentally* by elevating hormone concentrations, documenting the phenotypic effects, and relating these to fitness. We hypothesize that if experimental elevation of a hormone in both sexes causes each to alter its phenotype, then each might respond to direct selection, and have the potential to influence the other's evolution through correlated responses. Mutual sensitivity in this view is interpreted as a physiological predictor of evolutionary response, analogous to a genetic correlation. In contrast, if only one sex is sensitive to a hormonal manipulation, then the insensitive sex is 'insulated,' leaving the sensitive sex freer to evolve independently according to costs and benefits accruing to it alone.

Progress to date.—Completed experiments conducted in the field 2000-2003 (Clotfelter et al. 2004) have shown that unlike males, females were not sensitive to T in their nest defense (Clotfelter et al. 2004). Unique to females, T delayed breeding (egg-laying) by an average of 1 day (and interfered with brood patch formation in captive females), but did not affect incubation behavior (70% vs. 65%, T- and C-females respectively)(Clotfelter et al. 2004). Like males, T-females elevate free CORT and respond more rapidly to handling than control females (stress response). Also like males, T-females have higher levels of CBG (Zysling et al. 2003) and mount a less robust cell-mediated immune response (wing-web swelling after PHA injection). T-females are also more aggressive towards an intruder female than controls (Zysling et al. 2003). In sum, traits related to survival (natural selection) are similarly sensitive in both sexes; those related to reproduction were not.

Specific research questions.—Does experimentally elevated T influence female attractiveness to males? Does it influence offspring care in the form of feeding nestlings? Does it influence offspring development?

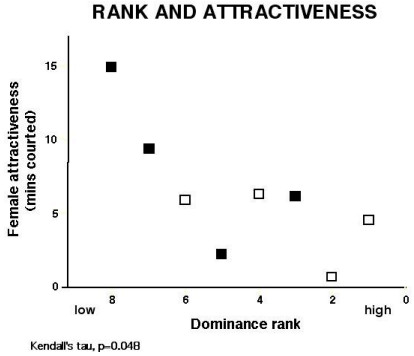
T enhances attractiveness in male juncos (Enstrom et al. 200x; Enstrom et al. 1997), and we will ask whether it also enhances attractiveness in females, has no effect, or reduces attractiveness. To distinguish, we will give males a choice between dyads of females consisting of one T-female and one C-female (implants as already described). Dyads will be matched for age, body size, and appearance and presented to males using the protocol of Enstrom et al. (1997) and Wolf et al. (2004)(observe females in absence of males for 30min, introduce male and observe female behavior for 20 min, release male into choice apparatus and record his behavior and amount of time he spends courting each female). Direct observation and videotapes will reveal differences in attendance time (time spent with T- or C-female) and female and male behavior.



In preliminary trials based on 30 males choosing between 5 sets of females (each male saw one of 5 possible female dyads constructed from 8 females and matched for age and body size), we found that males tended to direct more courtship towards C- than T-females (matched pairs, t-test, p=0.07)(Fig. 5, time spent with T-females in black, C-females clear)(Parker-Renga et al. 200x). These trials were based on too few females to be conclusive or to properly quantify the effect of T-implants on female behavior, but preliminary observations indicated that T-females tended to be more male-like in their behavior: they consumed more food, were more active, and some sang (ordinarily only males sing) (Parker-Renga et al. 200x). If T suppresses female attractiveness, we will conclude that enhanced T in females is likely to be deleterious with respect to sexual selection on females, particularly since we

know that females with more mates leave more offspring (Ketterson et al. 1998). If T has no effect, we will conclude that male attractiveness is not constrained by consequences that correlated responses to selection might have for female attractiveness. If T enhances female attractiveness, we will conclude that there is potential for reinforcement.

Female social status, does attractiveness trade-off with priority of access to resources?— An extremely interesting outcome of this preliminary experiment was the finding that females that were more attractive to males achieved lower social status when housed as a group with other females, while females that were unattractive were dominant (Fig. 6). The finding was based on only 8 females for which we correlated the amount of courtship time they received in the mate choice trials with the status they achieved when housed together after the mate choice trials were complete. The more attractive the female, the lower the status she achieved. In addition females treated with T appeared to be of higher status, though there were exceptions. Obviously this is based on a sample of 1 (1 flock), and we propose to test this further.



We will present a male with a dyad of two females and quantify the amount of courtship he directs toward each member of the dyad. We will then introduce the females into a neutral arena and determine which is dominant. The females will not have had prior social encounters with one another. We will sample the females for hormones as we complete the trials. We will assess members of 25 dyads of females as preferred/non-preferred by males and dominant/subordinate. A test of independence will reveal whether attractiveness and status are related. We will also compute mean time each female was courted by the male and compare dominant and subordinate individuals (matched-pairs). A significant difference will suggest a within-sex trade-off between traits related to sexual and natural selection, i.e., attractiveness vs. aggression.

Does experimentally elevated T suppress parental behavior in females as it does in males?—In males T reduces feeding of nestlings in the field, and we will determine whether the same is true of females. If females are also sensitive to elevated T, negative fitness consequences would be predicted, and males may be constrained. However, given the fitness cost likely to be entailed (Lynn and Wingfield 2003), females may have evolved insensitivity to T in nestling feeding behavior, as they have in nest defense (Clotfelter et al. 2004), which would leave males freer in this regard to evolve higher levels of T. To distinguish between these alternatives we will use established methods to compare T- and C-females in the field for rates of feeding offspring (videotaping supplemented with observation, 2 90-min sessions, 1AM, 1PM on day 3 and day 6 of nestling life). We can observe up to 40 nests/year, but unpredictable rates of nest predation predict that this experiment will require at least 2 breeding seasons.

Testosterone may also have fitness effects that are unique to females because it is incorporated into egg yolk and can influence embryonic development. If offspring differ in traits related to fitness because of levels of yolk T, female T levels will be under indirect selection. To assess the impact of maternal T on offspring development and the potential for indirect selection on females, we propose to hand-rear offspring to compare the development of traits known to be related to fitness: body size and tail white, an attractive plumage trait. Male juncos have been shown to be under correlational sexual selection for body size and tail white, and both traits are genetically correlated between the sexes (McGlothlin et al. in press). If females with experimentally elevated T produce larger sons with whiter tails, indirect sexual selection would favor an increase in female T. Conversely, if T-

females produced smaller sons with less tail white, female T would face negative indirect selection. This work will be done in collaboration with graduate student Joel McGlothlin. Young of T- and C-females will be taken from the nest between days 8-10 and hand-reared (see methods; also recall that T-females produce eggs with greater levels of yolk T than C-females). Tail development is complete ~2 weeks later, and we will compare tail white in a male and female offspring from each brood according to treatment of the mother, using standard statistics. As with parental behavior we can hope for up to 40 broods/year, but unpredictable rates of nest predation predict that this experiment will require at least 2 breeding seasons. [Note: we will transport these birds to Bloomington and later compare their response to GnRH as adults (Objective #3).]

Objective 2: To determine fitness consequences of experimentally elevated T for females in the field.

Rationale.—Traits that are sensitive to elevated T in both sexes but are beneficial in only one sex provide evidence of constraint. Traits beneficial in both provide evidence of reinforcement. Prior work has shown higher fitness in T-treated males, owing to greater success at extra-pair fertilizations (sexual selection) (Reed et al. 200x). The proposed experiments will provide evidence of adaptive or maladaptive outcomes of elevated T in females.

Progress to date.— Clearly a number of the traits that are sensitive to T in females could also have an impact on fitness, e.g. enhanced aggression or reduced immune function, and we have begun to compare T- and C-females for clutch size, frequency of young sired by extra-pair matings, daily survival rate of nests against predation, and proportion of females returning to breed the year following experimental treatment. To date we have found no effects on clutch size or egg dimensions (Clotfelter et al. 2004), but preliminary data indicate that T-treated females may be more subject than controls to nest predation while incubating [Mayfield daily survival rate of nests, 0.783 in T-females, 0.937 in C-females, p=0.049).

Specific research question.—Is female fitness enhanced, reduced, or unaffected by T-treatment?

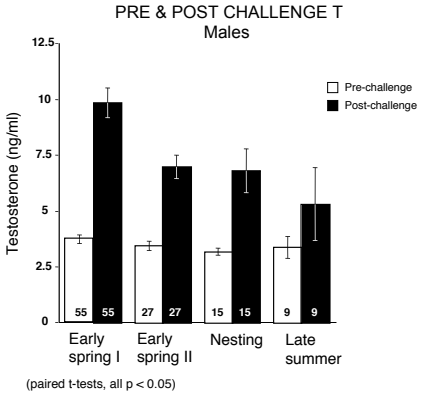
Proposed research.—We will compare T- and C-females for performance in the field on various measures of fitness, including number and quality of young produced and proportion of young sired by extra-pair males EPFs)(Ketterson et al. 1998; Raouf et al. 1997; Reed et al. 200x). We can readily monitor 40 females per year, each of which will initiate reproduction repeatedly, and 40% of which we expect to return. We will combine newly collected data in 2005-2007 with those collected in 2000-2002. The gap during which we have been measuring endogenous levels of T (see below) will allow us to begin with a fresh population of females that has not previously been zimplanted.

If T interferes with parental behavior (Objective #1), production should be lower in T- than C-females. If T enhances attractiveness (Objective #1), EPFs may be more frequent among T-females; if it reduces attractiveness, EPFs may be more frequent among C-females. We will also estimate female survivorship in the field by calculating simple return rates (percentage of females nesting in one year that nest in the next), the software MARK to estimate survival/failure, and lambda to estimate fitness (Reed et al. 200).

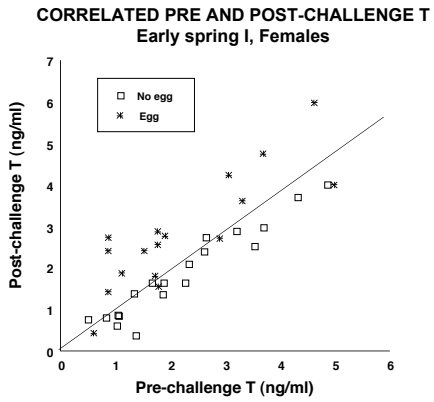
Objective 3: To assess individual variation in hormonal responsiveness and the potential for correlated response to selection.

Rationale.—As summarized above and in Ketterson et al. (200x), studies of natural populations demonstrate correlations between plasma T and fitness-related characters , and studies of domesticated species reveal both heritable variation in response to a GnRH challenge and correlated responses in females to selection on males. Our goal here is to extend our understanding of natural variation in T in male and female juncos.

Specific research questions.—Does individual variation in response to GnRH co-vary with incubation behavior, feeding of nestlings, plumage coloration, yolk steroid levels, or levels of IgG? Does response to GnRH predict frequency of extra-pair matings or co-vary with other measures of fitness? Do relatives resemble one another in their response to GnRH?



Progress to date.— To determine the proper dose of GnRH to stimulate T, presumably via an increase in LH, we compared the effect of 1.25 ug and 2.5 ug GnRH (Sigma L-3607) on 10 males by assaying pre- and post-injection plasma samples for T (EIA, Assay Designs, Casto et al., unpublished data). The response peaked at 30 min and declined to baseline levels by 60 min. We also injected 8 females with 1.25 ug and bled subsets of them at 15 min intervals, but they did not respond, apparently because they were not laying eggs at the time (see below). A collaboration begun with George Bentley, UC, Berkeley, will allow us to determine plasma levels of LH prior to and after a GnRH challenge in both sexes and to characterize variation at that level as well.



During 2003 and 2004, we challenged 131 males and 96 females in the field. The birds were caught in early spring, bled (50 ul blood), challenged (1.25 ug GnRH in 50ul buffered saline), bled again after 30 min, and released. Sub-sets of these birds were challenged again later in the spring, when feeding nestlings, and at the end of the breeding season. We have analyzed the 2003 data and found that males elevate T ~3-fold in early spring, and that the response declines seasonally (Fig. 7). In addition, individuals respond consistently in repeat challenges ($r=0.704$, $n=26$, $p < 0.001$), and response declines with age (younger males > than males older that 4 years)(data not shown).

Females also varied widely in baseline T, and pre- and post-challenge levels are correlated ($r=0.825$, $n=38$, $p=0.00$)(Fig. 8), indicating consistent individual variation. T increases in response to GnRH, but only when females are laying eggs (compare asterisks, which represent laying females to squares of non-laying females in Fig. 8). Hence for that variable we will need to study laying females.

GnRH, phenotype, and fitness.—In 2003 and 2004, we also used established methods (see above) to measure plumage coloration (129 males, 110 females), natural levels of immunoglobulins (IgG, EIA of plasma, n=116) (Demas and Nelson 1996), mate-feeding by males and incubation by females (n=45 videotapes), and rate of feeding of nestlings by both sexes (by temporarily removing 1 member of pair and measuring the feeding rate of other, then vice-versa, n=41 males, 31 females)(Clotfelter et al. 2004)(McGlothlin et al. prep.). In addition, we collected a freshly laid egg

from a subset of challenged females (n=38) and bled adults and young to obtain DNA for assessing parentage. These data will allow us to correlate hormone, phenotype, and fitness.

We are currently conducting hormone assays from 2004 and analyzing data from the field. The DNA has been extracted (n=547, 2003-04). Wing length and tail white are known to be under significant correlational sexual selection in males and genetically correlated with females (McGlothlin et al., in press), so it is promising to find that males with higher pre-challenge levels of T tend to have longer wings (p=0.06), which suggests that T and wing length may also have a correlational effect on mating success. In females, we will soon learn whether GnRH response co-varies with yolk steroids (yolk and response measured in relation to separate clutches) and parental behavior (incubation, nestling feeding).

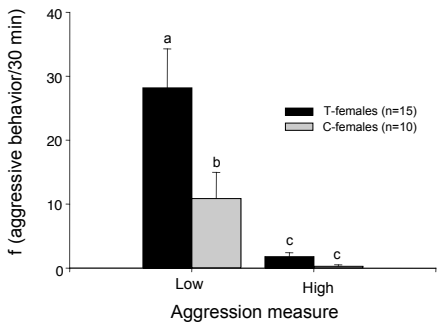
With respect to fitness, T-implants increase male success at EPFs (Raouf et al. 1997; Reed et al. 200x), and we will ask whether individuals with higher baselines levels of T or that respond more strongly to GnRH also have greater mating success and more EPF young. We are well along in genotyping (2003 nearly finished, 2004 has just begun) and when it is complete, we will employ the software package CERVUS (Slate et al. 2000) to determine parentage. Past experience leads us to expect 20-25% extra-pair young, no conspecific brood parasitism, and increased fecundity with increasing numbers of mates in both males and females (Ketterson et al. 1997; Raouf et al. 1997; Reed et al. 200x). We will ask whether males and females that give a strong response to GnRH differ in fecundity (fledglings/season) or probability of survival based on mark-recapture from year to year analyzed with the program MARK (Reed et al. 200x). If yes, we will be able to link results on natural variation in T and fitness to the results to be obtained from the experimental manipulations.

We have one goal beyond our primary goal of completing the analyses described in the last sections, which is to assess similarity of relatives in baseline T and response to GnRH. We will transport the young reared as part of Objective 2 to Bloomington, raise them to maturity, and assess response to GnRH the following year. Females and males will be established in aviaries and permitted to breed. Females will be challenged the day they lay egg 1 and males on the same day. Preliminary data collected in 2004 on 9 aviary females showed an increase in T in response to a GnRH challenge on the day they laid egg 1, so we know the method works on captives. The response was significant if we eliminated 1 outlier. We will compare the level of resemblance in response to GnRH in relatives and non-relatives.

Objective #4: To assess sexual independence by comparing T's role in the activation of aggressive behavior in males and females.

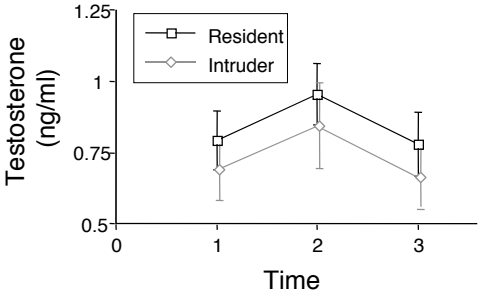
Specific research question.—Is the aggressive response by a captive resident female to an intruding, same-sex conspecific mediated by T in females, or does female aggression co-vary with some other hormone (or both)?

Progress to date.--Using a resident-intruder paradigm, in which we measured the behavioral response of captive females to an intruder, we found that T-implants increased a resident's level of aggressive response over that of controls (Zysling et al. 200x; Zysling et al. 2003). Females were held in large outdoor cages, implanted as T- or C-, and exposed to an intruder. Aggressive behavior was quantified over a 30 min period and was more frequent in the T-females (see Fig 9).



In a second experiment on un-implanted females, in which females were bled, then housed with a male for 24 h and bled again, then exposed the next day to an intruder for 30 min and bled a third time, resident females were always aggressive towards intruders. However the T-levels of residents and intruders did not differ or increase after the 'challenge' (Fig. 10). The females formed dominant-subordinate relationships, and importantly, only dominant females bred (built nests and laid eggs), not subordinates (Jawor et al. 200x).

Considering both experiments together, we conclude that prolonged exposure to T can enhance aggression in females (Expt. 1), but that T may or may not mediate female aggression because it is not elevated in females after a single intrusion (Expt. 2). We also conclude that female-female aggression may predict reproductive success because it can suppress reproduction (Expt 2).



Proposed experiments.— We propose to determine whether females are simply less sensitive than males to social challenges and require more stimulation before they increase T, perhaps because T has disadvantages and provides a benefit only when intrusions are persistent. To determine whether this is the case, we will conduct a challenge experiment like Expt 2, in which females are exposed to repeated intrusions to determine whether T rises after repeated challenges. In the second proposed experiment, we will repeat the challenge experiment as originally conducted but measure other hormones instead, E2, DHEA and P.

Do resident females elevate T in response to repeated intrusions? On day 1 we will draw 6 females from separate all female flocks (to avoid the influence of prior relationships among the females) and place them in individual cages. One female, chosen at random, will be designated as the resident and will be bled for later determination of circulating levels of T. Also on day 1, a male will be introduced into a breeding aviary. On day 2, the resident female will be introduced to the breeding compartment to form a pair with the male and bled again: the other females will remain in their individual cages. For half the residents, to be known as the Experimental-residents, each half-day over the next 2.5 days, we will introduce a different female for 1 hour in the AM and 1 hour in the PM. On day five, after the fifth female has been present for 1 hour, we will capture the resident female and bleed her again. For the other half of the residents, to be known as the Control-residents, on each half-day we will enter the breeding aviary, place an empty cage in the compartment, and remove it after 1 hour. When the experiment is complete, we will compare the 2 pre-challenge levels of T with the post-challenge levels in a repeated measures 2-way ANOVA that will also compare Experimental- to Control-residents. Also after each introduction we will also quantify the frequency of aggressive encounters. We predict that if T plays a role in prolonged and intense female-female competition and aggression, T will rise in repeatedly challenged experimental females. If not, some other hormone may mediate female aggression.

Do females elevate E_2, DHEA or P in response to a standard 30 min intrusion? We will use the same design as the previous Expt. 2 (Jawor et al., submitted). That is, we will assess hormone levels before and after pair-formation and after introduction of an intruder, but measure additional hormones. We will draw 2 females from separate all-female flocks, bleed them for later analysis of

hormones, and place them in individual cages. One female, chosen randomly, will be the resident; the other will be the intruder. Also on day 1, we will introduce a male into a breeding aviary. On day 2 the male and the resident female will pair. On day 3 we will introduce the intruder to the resident and after 30 min of interaction quickly capture and bleed the resident and intruder females. Because the time course the experiment is short, 2 days between bleedings, and the volume of plasma needed for the assays of E2, P and DHEA is greater than what was needed for the EIAs used to measure T, we will run separate trials. After 12 randomly chosen trials of the 24 planned, we will measure the change in female E_2 and P in before and after the intruder was introduced. After the other 12 trials, we will measure the change in DHEA. We will test significance using repeated measures ANOVA.

Interpretation.—From the first experiment we will learn whether or not prolonged and repeated exposure to intrusions stimulates females to elevate testosterone as males do. From the second we will learn whether E2 and DHEA rise under conditions that are known not to elevate T. If we find that T is not related to intrasexual competition, we will conclude males and females may rely on different mechanisms, which would indicate sexual independence. Future experiments would pursue how aggression is mediated in females.

Putting it all together.—Research described in this proposal will uncover the extent of resemblance between the sexes in their phenotypic sensitivity to testosterone, which in turn will predict the potential for both direct and correlated responses to selection (Fig. 1). Traits that prove sensitive to an increase in T in only one sex will be predicted to evolve independently; traits that prove sensitive in both sexes may respond in correlated fashion. If the sensitive traits are beneficial in one sex and detrimental in the other, they will provide evidence of constraint. Alternatively, the sensitive traits that have fitness consequences in only one sex but are neutral in the other will indicate sexual independence. Whether or not the sexes are hormonally correlated in natural variation in T and response to GnRH will provide independent insight into the level of evolutionary interdependence between the sexes. Finally, the hormonal mediation of aggression will serve as a case study to explore sexual inter- 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.

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 devoted to members of groups underrepresented in science http://www.indiana.edu/~animal/academics/reu.html and has just been renewed for four years. 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. Potential societal implications of research that would be supported by this proposal include implications for (1) impact of endocrine disrupting chemicals in the environment, (2) improved methods for breeding captive songbirds for conservation, and (3) a greater understanding of the relationship between sex and gender.