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Testosterone and innate immune function inversely covary in a wild population of breeding Dark-Eyed Juncos (*Junco hyemalis*)

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Summary

1. Innate immunity refers to the non-specific components of the primary immune response, which act broadly to destroy pathogens. Effective innate immune responses may save an individual the energetic costs associated with activating subsequent specific immune responses.

2. Testosterone can suppress immune function *in vitro* and *in vivo*. Most studies examining testosterone's effects on immunity have focused on experimentally elevated testosterone and acquired immune responses (e.g. humoral and cell-mediated responses to foreign antigens). Few studies have investigated the relationship between endogenous levels of testosterone and innate immunity.

3. In a wild breeding population of Dark-Eyed Juncos (*Junco hyemalis* Linnaeus), we asked whether endogenous levels of testosterone measured at several points during the breeding season covaried with two components of innate immunity: total levels of non-specific immunoglobulin-G (IgG), and complement levels.

4. Testosterone levels were significantly negatively correlated with both total IgG and complement activity. Both immune measures were also positively correlated with body mass. Taken together with experimental results from the same species, these results suggest that elevated testosterone levels may compromise innate as well as acquired immune function.

Key-words: Breeding season, complement, antibodies, hormone, IgG, songbird

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Introduction

Although numerous studies have induced suppression of the immune system by elevating testosterone (T) experimentally (e.g. Duffy *et al*. 2000; Peters 2000; Casto *et al*. 2001; Mougeot *et al*. 2004), the literature regarding whether elevations in endogenous T are immunosuppressive is conflicting (Saino, Møller & Blozern 1995; Hasselquist *et al*. 1999; Greenman, Martin & Hau 2005). A recent meta-analysis provided limited support for the immunosuppressive effect of T in some species, but concluded that the question is still open (Roberts, Buchanan & Evans 2004). The pathways by which T might affect immune function are also controversial; T may either bind directly to androgen receptors within the immune system (Tanriverdi *et al*. 2003) or assert its effect via indirect pathways such as glucocorticoid release (Casto *et al*. 2001; Owen-Ashley, Hasselquist & Wingfield 2004).

To date, most studies examining the relationship between T and the immune system have focused on humoral and cell-mediated immune responses (e.g. Hasselquist *et al*. 1999; Casto *et al*. 2001), which serve, in part, to generate immune memory that protects the animal during a subsequent encounter with a specific pathogen. Mounting a specific immune response however may be energetically costly (Lochmiller & Deerenberg 2000). Another important component of the immune system is innate immunity, which provides a first line of defence against a wide range of pathogens, working to neutralize them before a specific immune response is triggered (Mayer 1948; Nielsen *et al*. 1978; Whitelaw *et al*. 1979; Frank, Miletic & Jiang 2000; Ochsenbein & Zinkernagel 2000; Rickert 2005). Innate immune function may be particularly important in determining the survival of an animal upon its first encounter with a disease, and a successful innate response may help avoid

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a costly specific response (Lochmiller & Deerenberg 2000). To our knowledge, only one study has examined the relationship between T levels and non-specific immune defences (lymphocyte counts) and it was conducted on a highly polygynous and strongly dimorphic species, the Jungle Fowl (*Gallus gallus*, Zuk, Johnsen & Maclarty 1995).

The goal of this study was to assess the relationship between endogenous T levels and innate immune function in a socially monogamous songbird, the Dark-Eyed Junco (*Junco hyemalis* Linnaeus). Previous work on juncos has shown that experimentally enhanced T decreases acquired immune responses as well as survival (Casto *et al*. 2001; Reed *et al*. 2006; Zysling *et al*. 2006). Whether or not these measures covary naturally in the wild is not known. In the present study we measured endogenous T levels in wild breeding males, as well as two components of innate immune function: circulating immunoglobulin G (IgG) and complement lysis. All measurements were obtained from adult males at multiple stages of breeding to control for potential seasonal variation. We predicted that males with higher endogenous T would show lower innate immune function. Such a result, in combination with the previously demonstrated suppressive effects of experimentally elevated T on specific immune function would suggest that T acts, directly or indirectly, as a general suppressor of immune function in juncos.

Materials and methods

CAPTURE AND COLLECTION OF BLOOD SAMPLES

Fifty-four male Dark-Eyed Juncos, ranging in age from 1 to 7 years, were captured between 21 April and 5 August 2004 near the University of Virginia's Mountain Lake Biological Station (37°22′ N, 80°32′ W), Giles Co., VA, USA. Seventy-nine blood samples were collected at four different stages of the breeding season to assess T and innate immunity. Collecting multiple samples allowed us to control for within-individual variation across the breeding season and to examine the consistency of individual responses. First captures of each bird were by baited mist nets and potter trips and took place between 21 April and 11 May ($n = 45$) and are referred to as Early Breeding A. A subset of these birds ($n = 11$) was captured and sampled a second time during the early season between 1 and 13 May during a period defined as Early Breeding B. The mean time elapsed between first and second captures was 11 days (range = 8–21 days). During the dates encompassed by Early Breeding A and B, pair bonds have typically formed, but males are frequently observed singing and mate guarding (Nolan *et al*. 2002). Females also begin to lay eggs and incubate clutches during this period (date of first egg in 2004, 25 April). Males were also caught at their nests when feeding nestlings that were 6–8 days old (Nesting, 20 May–20 July, $n = 14$). During the late breeding season, when most nesting was complete, males were caught again using baited mist nets (Late Breeding, 20 July–5 August, $n = 9$). Eight males were sampled three times, 9 males were sampled twice, and the remaining 37 were sampled once. Sampling occurred during both the morning and evening during Early Breeding A (mean capture time [range]: am [$n = 28$] = 09.27 [07.30–11.50]; pm [$n = 17$] = 19.42 [18.05–20.30]) and Early Breeding B (mean capture time [range]: am [$n = 8$] = 09.12 [06.16–11.25]; pm [$n = 3$] = 20.07 [19.48–20.30]); sampling during Nesting and Late Breeding occurring during the morning (mean capture time [range]: 07.56 [06.30–09.40] and 09.43 [08.25–10.45], respectively). Time of sampling (am *vs* pm) did not affect testosterone levels (linear mixed model: $P = 0{\cdot}95$) and there was no interaction between breeding stage and sample time ($P = 0{\cdot}29$).

Upon capture, birds were transported to the laboratory, where they were banded (if necessary), morphometric measurements (wing length, tail length, tarsus [± 0·1 mm] and mass [± 0·1 g], using a Pesola spring balance) were taken and age was estimated using plumage and iris coloration (Nolan *et al*. 2002). Age in years was determined from capture records from previous years. A blood sample (~125 µl) was collected from the alar wing vein in heparinized microcapillary tubes. Blood samples were centrifuged to separate plasma, which was then stored –20 °C until assays were performed. Approximately 25 µl of plasma were allocated for the immune measures, and the remainder was used to measure T concentrations. Birds were returned and released at the site of capture.

All animal capture and handling procedures have been approved by the Bloomington Institutional Animal Care and Use Committee at Indiana University.

IMMUNOGLOBULIN ASSAY

IgG is the most abundant immunoglobulin in circulation and can be measured without any prior manipulation of an individual's immune system (e.g. an immune 'challenge'), and may represent a state of immunological 'readiness.' Total IgG levels in plasma were determined using a sandwich enzyme-linked immunosorbant assay (ELISA) with slight modification to a previously described protocol (Demas & Nelson 1996). Ninety-six well immunoplates (Nunc®, VWR, Chicago, IL) were coated with 100 µl well^{-1} of donkey polyclonal antibody against chicken IgG (Jackson laboratories, West Grove, PA) diluted in carbonate/bicarbonate buffer (0·1 M, pH 9·6) to 3 µg ml^{-1} and incubated overnight at 4 °C. The following day, plates were washed three times with PBS (0·05 M, pH 7·4) containing 0·05% Tween-20 and 0·001% NaN_3 using an automatic microplate washer (Bio-Rad, Model 1575, Hercules, CA). Plates were then coated with 200 µl milk blocking buffer (5% non-fat dry-milk [SACO®, Middleton, WI] diluted in PBS), incubated at room temperature for 2 h, and then washed. Next, plasma samples were diluted 1:20 in PBS and added in duplicate to the plates. A plate positive control was

prepared using a mixed-sex pool of plasma obtained from Dark-Eyed Juncos housed at the Indiana University Kent Farm Bird Observatory, Bloomington, IN. The pooled plate positive was diluted 1:20 in PBS and then added to the plate in triplicate, and the plate was incubated overnight at 4 °C. Plates were washed the following day and 100 µl of a 1:500 dilution of rabbit anti-starling IgG (courtesy of G. F. Ball & R. J. Nelson) were added to all wells as a capture antibody and incubated at 37 °C for 1 h. The plates were washed and 100 µl of alkaline Phosphatase-conjugated goat antirabbit IgG diluted 1:500 in PBS were added to each well. Plates were again incubated for 1 h at 37 °C and subsequently washed. Some 100 µl of substrate buffer (0·1 M *p*-nitrophenyl phosphate in diethanolamine buffer [0·1 M, pH 9·5] containing 5 mM $MgCl_2$) was added to each well. Plates were incubated at room temperature for 20 min and then optical density of the resulting coloured product from each well was measured at 405 nm with a microplate reader (Bio-Rad, Hercules, CA). Sample concentrations of IgG were determined as a percentage optical density of the controlled pooled-plate positive.

HAEMOLYTIC COMPLEMENT ASSAY

Complement is a non-specific component of innate immunity, which consists of a group of proteins with the ability to lyse pathogenic cells (Mayer 1948). Complement activity in plasma was measured on a subset of 32 samples for which there was sufficient plasma. Sixteen samples were from Early Breeding A, 3 were from Early Breeding B, 10 were from Nesting and 3 were from Late Breeding. These samples were from 24 different males; 1 of these was sampled 3 times, 6 were sampled twice and 17 were sampled once. Methods used are described by Mayer (1948), with modifications by Sinclair & Lochmiller (2000) and De Wall *et al*. (1988). Briefly, we added in duplicate 5 µl plasma, diluted 1:40 in dextrose–gelatin–veronal buffer (VB) (BioWhittaker, Walkersville, MD) to a round-bottomed microtitre plate, and then serially diluted samples twofold. Next 25 µl of a 0·6% suspension of washed sheep red blood cells (SRBC, MP Biomedicals, Irvine, CA) and 25 µl of a 1:40 dilution of rabbit anti-SRBC antibody were added to the sample wells. Lysis wells of 0% and 100% were created by adding 65 µl VB or water, respectively, and 25 µl of 0·6% washed SRBC. The microtitre plate was then sealed and gently shaken for 5 min using a plate shaker and incubated at 37 °C for 90 min. The plate was then centrifuged for 5 min at 500 r.p.m. at room temperature to separate un-lysed SRBC at the well bottom from the supernatant containing lysed cells. Some 60 µl of supernatant were then transferred to a new microtitre plate and absorbance was measured at 405 nm with a microplate reader (Bio-Rad). Haemolytic-complement activity was expressed as CH_{50} units ml^{-1} plasma, where 1 CH_{50} unit equals the amount of complement required to lyse 50% of the SRBC in culture (Mayer 1948). Intra-assay variation was less than 5%.

TESTOSTERONE ASSAY

Plasma T concentrations were determined using a commercial enzyme immunoassay (EIA) (Assay Designs, Inc., #901–065, Ann Arbor, MI). The kit has low (7·2%) cross-reactivity with androstenedione and negligible (<1%) cross-reactivities with dehydroepiandrosterone, oestradiol, dihydrotestosterone, progesterone and corticosterone. Procedures followed Clotfelter *et al*. (2004). Briefly, plasma samples were diluted six-fold with distilled water, then ~2000 c.p.m. of [3H] T (NET-553; New England Nuclear Corp., Boston, MA) was added to allow calculation of recoveries, and extracted twice. Extracts were re-suspended in 50 µl ethanol and diluted to 350 µl with assay buffer. Some 100 µl were used to determine recovery percentages and 100 µl were added in duplicate in the EIA. T concentrations were determined with a 4 parameter logistic curve-fitting program (Microplate Manager; Bio-Rad) and corrected for incomplete recoveries. Intra-assay variation, which was calculated as the coefficient of variation of values obtained from standard samples of known concentration, ranged from 3·01 to 8·72%; interassay variation was 8·46%. In order to reduce interassay variation, T concentrations were adjusted by a correction factor for each plate equal to the mean of the standards on a given plate divided by the grand mean of all standards.

STATISTICAL ANALYSES

Linear mixed models were used to examine the relationships between immune parameters and T. This type of model can account for repeated measures on the same individual while allowing for unbalanced sampling across time points (Verbeke & Molenberghs 2000). The raw values of total IgG and complement activity were natural log (ln) transformed before analysis to improve normality of the residuals and each was used as the dependent variable in separate models. Each model included stage (Early Breeding A, Early Breeding B, Nesting or Late Breeding) as a fixed effect, plasma T concentration (ln-transformed), age (in year), mass (g), tarsus (mm, average of all measures taken across the breeding season) and handling time (defined as the time in minutes between capture and the collection of the blood sample, ln-transformed; mean elapsed time = 34·37 min; range 3–105 min) as covariates, and individual identity as a repeated random effect. Non-significant effects were removed from the final model using backward elimination with a removal criterion of $\alpha > 0{\cdot}2$, and AIC values were compared to determine the best final model. Parameters were estimated using restricted maximum likelihood (REML). The covariance structure used for the repeated measures was factor-analytic first order, which allows for heterogeneity of both variances and covariances with the estimation of a minimum number of parameters (number of repetitions + 1). In addition, the final models were run a second time using a compound symmetrical covariance structure, allowing

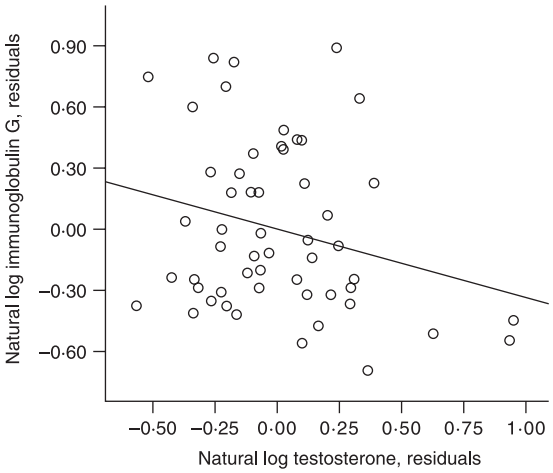

Fig. 1. Immunoglobulin G concentration (% of plate positive, natural log-transformed) in relation to plasma testosterone concentration (ng ml^{-1}, natural log-transformed). Each data point represents a single individual. Multiple measurements from an individual were summarized for presentation using estimated marginal means from ANOVAS that included breeding stage and individual. Plotted residuals and line of best fit are from a regression of these estimated marginal means that included the continuous variables in the final model (Table 1).

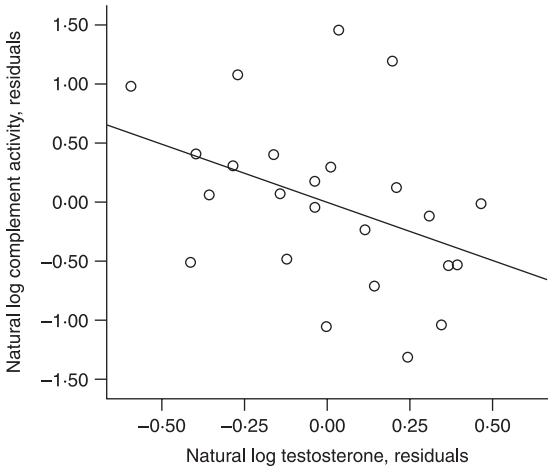

Fig. 2. Complement activity (CH_{50} units ml^{-1}, natural log-transformed) in relation to plasma testosterone concentration (ng ml^{-1}, natural log-transformed). Multiple measurements were summarized as in Fig. 1.

estimation of the intraclass correlation coefficient, a measure of individual consistency (or repeatability) of response. Although analyses were performed using all data, figures display residuals and single points for individual birds for ease of interpretation. All analyses were performed using SPSS 14 for WINDOWS (SPSS Inc., Illinois).

Results

Plasma T was significantly negatively correlated with both total IgG and complement activity (Table 1; Figs 1 and 2). Body mass was positively correlated with both IgG and complement activity, and tarsus was positively correlated with complement activity (Table 1). Other covariates were removed from the models ($P > 0{\cdot}2$).

IgG varied significantly with stage of breeding but complement activity did not (Tables 1 and 2). A comparison of the estimated marginal mean values for each breeding stage revealed that ln IgG was lower in Early Breeding A (mean ± SE = 4·53 ± 0·064) than in Early Breeding B (4·87 ± 0·112; $P = 0{\cdot}010$) and lower in Late Breeding (4·37 ± 0·132) than in Early Breeding B ($P = 0{\cdot}006$) and nestling feeding (4·73 ± 0·118; $P = 0{\cdot}049$). The probability of obtaining three out of six significant tests by chance at $\alpha = 0{\cdot}05$ is 0·002 (Bernoulli equation, Moran 2003).

Table 1. Linear mixed models of immunoglobulin G (IgG, natural log-transformed) and complement activity (CH_{50}, natural log-transformed). Repeated measures were modelled using a factor analytic, first-order covariance structure. Final models after backward elimination are presented below

	ln IgG (% plate positive)				ln CH_{50} units ml^{-1}			
Fixed effects	Estimate	df	*F*	*P*	Estimate	df	*F*	*P*
Stage		3, 27·0	3·99	0·018		3, 3·24		0·116
ln testosterone	–0·32	1, 59·9	4·73	0·034	–1·48	1, 1·56	160·61	0·015
Mass	0·07	1, 54·3	4·15	0·047	0·21	1, 2·07	19·38	0·045
Tarsus					0·63	1, 1·70	63·50	0·024

Table 2. Mean ± SE values for testosterone (T), immunoglobulin G (IgG), mass and complement (CH_{50}) during different stages of the breeding season

Breeding stage	T (ng ml^{-1})	IgG (% plate positive)	Mass (g)	CH_{50} units ml^{-1}
Early Breeding A	3·05 ± 0·199	103·92 ± 7·653	22·24 ± 0·204	0·49 ± 0·082
Early Breeding B	3·10 ± 0·303	137·96 ± 16·294	22·24 ± 0·500	0·92 ± 0·304
Nesting	2·83 ± 0·224	113·90 ± 14·922	20·92 ± 0·336	0·91 ± 0·249
Late Breeding	2·75 ± 0·112	83·19 ± 11·200	21·44 ± 0·430	0·48 ± 0·100

When controlling for the fixed effects in the final model, IgG showed positive but non-significant within-individual repeatability across stages ($r = 0{\cdot}33$, Wald $Z = 1{\cdot}67$, $P = 0{\cdot}09$). Complement did not show significant repeatability, perhaps because of the low number of individuals from which there were repeated measurements ($r = -0{\cdot}24$, Wald $Z = -0{\cdot}36$, $P > 0{\cdot}1$).

Discussion

In the present study, we found significant negative correlations between endogenous concentrations of T and two components of innate immunity. In addition, we found significant positive correlations between both innate immune measures and body mass. Tarsus length, a measure of skeletal body size, was positively correlated with complement, but not with total IgG. Together, these findings suggest potential trade-offs among mating effort, energy stores and innate immune function. We also found that circulating IgG levels differed significantly among breeding stages, even after controlling for T and body mass.

Because the innate immune system acts as an immediate response to pathogens, it may be particularly important for survival in the wild (Mayer 1948; Nielsen *et al*. 1978; Whitelaw *et al*. 1979; Frank *et al*. 2000; Lochmiller & Deerenberg 2000; Ochsenbein & Zinkernagel 2000; Rickert 2005). The components measured in this study, circulating antibodies and complement, work together to neutralize a wide variety of pathogens, including West Nile virus (Mayer 1948; Ouma *et al*. 1997; Frank *et al*. 2000; Mehlhop *et al*. 2005). Suppression of the innate immune system may further decrease survival by way of interactions with the acquired immune system. An innate immune response may help activate humoral and pro-inflammatory (cell-mediated) immune responses if needed (Pastoret *et al*. 1998; Frank *et al*. 2000; Carrol 2004) or, if successful, may allow an animal to avoid some of the energetic costs that would be associated with such specific immune responses (Lochmiller & Deerenberg 2000; Eraud *et al*. 2005).

Given that juncos with lower circulating IgG and lower complement activity had higher levels of testosterone, the question of cause and effect arises. Is higher testosterone the cause of lower immune function as predicted by the immunocompetence handicap hypothesis (Folstad & Karter 1992), or did some factor that stimulated an increase in immune function also drive down testosterone? Earlier studies reporting an inverse relationship between natural concentrations of immunoglobulins and T have been unable to resolve this question, because they could not ascertain whether individuals with high levels of IgG are better prepared to ward off a pathogen they might encounter in the future, or alternatively, recovering from a recent or prolonged infection (e.g. Saino *et al*. 1995). Complement activity, in contrast, is not known to increase in response to infection (Olaho-Mukani, Munyua & Njogu 1995; Benjamini, Sunshine & Leskowitz 1996; Ouma *et al*. 1997). Consequently, the inverse relationship between T and complement reported here is more consistent with the conclusion that T suppresses innate immunity than it is with the explanation that recent infection or innate immunity suppresses T.

Correlative data alone cannot confirm such a link, but a previous study of this same population of juncos found that experimentally elevated T reduced both humoral (in response to sheep red blood cells) and cell-mediated (in response to a challenge of phytohaemagglutinin) immunity (Casto *et al*. 2001). Thus, the current data, combined with these previous findings, suggest that elevated T likely imposes costs at multiple levels of the immune system in juncos, a conclusion that is strengthened by the observation that experimentally elevated levels of testosterone decrease survival rates of male juncos in relation to controls (Reed *et al*. 2006).

If T does indeed suppress innate immunity, it may do so by way of a number of mechanisms. T may act directly on the immune system. Androgen receptors are found in both immune cells and organs in mammals (Tanriverdi *et al*. 2003). Studies in birds suggest that T may more often act via indirect pathways, for example by triggering the secretion of glucocorticoids, which are known to be immunosuppressive (Apanius 1998; Casto *et al*. 2001; Owen-Ashley *et al*. 2004). T may also divert energy from the maintenance of immune function by increasing other energetically costly behaviours or physiological responses (Demas & Nelson 1996; Lochmiller & Deerenberg 2000).

The positive correlations observed between mass and immune parameters suggest that energetic mechanisms may be important for determining innate immune function in juncos. Immune function is known to vary directly in response to energy availability in many species (Demas 2004). In this study, lighter males showed lower innate immune function, suggesting that individuals in poor energetic condition may thus face an increased cost of prolonged elevation of T. This relationship was significant when controlling for tarsus length, a measure of skeletal size that also covaried significantly with complement activity (but not IgG).

Antibody levels varied significantly across breeding stages, with the lowest levels observed during the earliest and latest stages. The causes of the seasonal variation are not known but may be due to environmental stressors such as cold weather in early spring or the energetic demands of establishing territories in early spring. The reasons for the decline in late summer are less clear; however, the energetic cost of moult during the late summer may contribute to this trend. Interestingly, parasite prevalence in many birds, including a different population of juncos (e.g. Deviche, Greiner & Manteca 2001), is highest late in the breeding season, a time when total IgG levels were at their lowest. In addition, in this population, the age (second-year adult or after second-year adult) of the bird had a significant affect on blood parasite prevalence, and the authors of this study attributed this to differences in testosterone levels between these age classes (Deviche *et al*. 2001). No effect

of age was found for either IgG or complement in the current study. This could be due to the lack of difference in testosterone levels observed between these age classes in our population during the period of our sampling (Jawor *et al*. in press). As we did not sample parasite prevalence, our results do not exclude the possibility that age may have an effect on parasite prevalence in our population.

Sexual advertising in male vertebrates is often mediated by the gonadal steroid hormone testosterone (T). T may enhance mating success by increasing attractiveness to females or success in male–male competition (Alatalo *et al*. 1996; Enstrom, Ketterson & Nolan 1997; Raouf *et al*. 1997; Foerster & Kempenaers 2005). T-mediated sexual advertising may act as an honest indicator of male quality because of survival costs, such as suppression of immune function, that may be associated with prolonged elevation levels of T (Folstad & Karter 1992; Wingfield, Lynn & Soma 2001; Casto *et al*. 2001; Reed *et al*. 2006). The correlational relationship between T and innate immunity reported here, combined with previous experimental manipulation of T in the same population of Dark-Eyed Juncos (Casto *et al*. 2001; Reed *et al*. 2006), suggest that T may act directly or indirectly to generate a survival cost by suppressing innate immunity, a result that is consistent with the predictions of the immunocompetence handicap hypothesis (Folstad & Karter 1992). Future work should examine the physiological pathways by which T affects innate immunity, as well as the fitness consequences of natural variation in innate immune function.

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