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## Testosterone and prolactin in two songbirds that differ in paternal care: the blue-headed vireo and the red-eyed vireo

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### Abstract

We investigated the hypothesis that species differences in paternal care in birds may result from differences in concentrations of circulating testosterone (T) and prolactin (PRL). Concentrations of plasma T and PRL were compared in breeding Blue-headed Vireos (*Vireo solitarius*) and Red-eyed Vireos (*Vireo olivaceus*), passerine congeners with biparental and maternal incubation, respectively. In male Blue-headed Vireos, plasma T remained low from prenesting to fledgling stages; whereas in male Red-eyed Vireos, plasma T was highest during prenesting and progressively decreased during incubation, nestling, and fledgling stages. In male Blue-headed Vireos, plasma PRL was similar to that in female Blue-headed Vireos and was higher than in male Red-eyed Vireos at all breeding stages. Plasma PRL increased in male Red-eyed Vireos at the incubation stage and remained moderately elevated through the nestling and fledgling stages. In male Blue-headed Vireos, the combination of high PRL and low T during the prenesting stage may promote brood patch formation and nest building, and, at later stages, incubation and feeding of young. In male Red-eyed Vireos, high PRL and low T during incubation and nestling stages may facilitate the feeding of young, as seen in males of other species. Our observations support the hypothesis that differences in paternal care reflect differences in circulating T and PRL.

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**Keywords:** testosterone; prolactin; parental care; vireos; *Vireo solitarius*; *Vireo olivaceus*

### Introduction

Paternal behavior in birds is usually characterized by low plasma testosterone (T) and elevated plasma prolactin (PRL): a peak in plasma T occurs early in the breeding season followed by a decrease at about the time PRL concentrations begin to rise (Ball, 1991). It is widely accepted that T stimulates male song (Nottebohm, 1981) and aggression (Wingfield et al., 1987) in birds. Further, if early breeding concentrations of plasma T are experimentally maintained into later stages of the breeding cycle, parental care is inhibited at those stages (Hegner and Wingfield, 1987; Oring et al., 1989; Ketterson et al., 1992; Schoech et al., 1998; but see Hunt et al., 1999; Van Duyse et al., 2000).

Increased plasma PRL is associated with incubation behavior, and the sex that assumes the role of primary incubator typically has greater relative plasma PRL concentrations (for reviews, see Goldsmith, 1991; Buntin, 1996).

Previous efforts to generalize across species about the relationship between the relative patterns of T and PRL and the form and frequency of male care have been complicated by species differences in natural history, mating system, and ancestry (Balshine et al., 2002). A few comparative studies of T and PRL have been performed with closely related species but because male parental behavior did not differ between them, not surprisingly, neither did patterns of plasma PRL (Williams and Sharp, 1993) or T and PRL (Hector et al., 1986).

In the few cases in which comparisons of plasma T and PRL have been made between males of species that differ in the relative amount of a parental behavior, hormone levels

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differed very little across breeding stages. Oring and colleagues compared T and PRL in two sex-role-reversed species, the Spotted Sandpiper (*Actitis macularia*), with predominantly male incubation (Oring et al., 1986; Fivizzani and Oring, 1986), and the Wilson's Phalarope (*Phalaropus tricolor*), with strictly male incubation (Oring et al., 1988). In males of both species, concentrations of plasma T declined, and PRL increased, with the onset of incubation. Gratto-Trevor and colleagues (1990a, 1990b) compared the monogamous Semipalmated Sandpiper (*Calidris pusilla*), a shorebird with shared incubation, to the polyandrous Red-necked Phalarope (*Phalaropus lobatus*), a shorebird with strictly male incubation, and observed no hormone differences that could be related to differences in parental behavior.

A comparison of T and PRL in species that differ in the form of male care has not been made. Specifically, a comparison between species that differ in the presence or absence of male incubation may offer new insight into the hormonal basis of incubation by males. Here we document plasma T and PRL in males of two closely related, sympatric species that exhibit the same social mating system but differ in the type of care provided (incubation) as well as the magnitude of care (feeding nestlings).

Blue-headed Vireos (*Vireo solitarius*) and Red-eyed Vireos (*Vireo olivaceus*) are socially monogamous species that breed sympatrically and have the same average clutch size, egg size, and body size (James, 1973; Cimprich et al., 2000, personal observation). Although male Red-eyed Vireos do not incubate eggs, they contribute to parental care by helping their mates provision nestlings (Kendeigh, 1952). In contrast, like many other members of the *Vireo* genus, male Blue-headed Vireos assist their mates with nest construction, daytime incubation of eggs, as well as the feeding of nestlings (James, 1973; Morton et al., 1998).

In male Red-eyed Vireos, we predicted that changes in concentrations of T and PRL during the breeding season would be similar to the commonly observed passerine pattern described above (Ball, 1991). In male Blue-headed Vireos, we predicted that, in general, plasma T would be lower and plasma PRL would be higher because they perform care at every breeding stage, and even in behaviors common to males of both species, like feeding nestlings, male Blue-headed Vireos perform greater rates of care. Further, we predicted that the seasonal transition from high T to high PRL would occur earlier in the breeding season in male Blue-headed Vireos because they participate in nest building and incubation, unlike male Red-eyed Vireos. Finally, because Blue-headed Vireo parents perform similar amounts of care at all stages of reproduction, we predicted males and females of this species would have similar plasma PRL concentrations. We predicted male and female Red-eyed Vireos would differ in their plasma PRL concentrations.

## Methods

At the University of Virginia's Mountain Lake Biological Station near Pembroke, Virginia (lat. 37°22'N, long. 80°32'W, elevation 1160 m), male Blue-headed Vireos arrive at the end of April and beginning of May, with the first few individuals arriving approximately mid-April. Male Red-eyed Vireos arrive during the second week of May, with the first few arriving during the first week of May. Every 2–3 days between 1 May and 7 August, we monitored vireo nests and observed nesting behavior.

We caught individuals and collected blood samples during four stages of the breeding season: prenesting (from arrival on breeding grounds until the onset of incubation), incubation (12–14 days for both species), nestling (12–14 days for both species), and fledgling (from 2 to 4 weeks after young leave the nest for both species). Both species make subsequent breeding attempts if the clutch is abandoned, lost due to predation, or successfully completed early in the season; therefore, pairs could be at any reproductive stage throughout the breeding season. Using mist nets and playbacks of conspecific song or Blue Jay (*Cyanocitta cristata*) song, we captured Blue-headed Vireos and Red-eyed Vireos and bled them immediately (see below). Sex was easily determined by the presence (male) or absence (female) of song during playback and later verified during behavioral observations. We recorded the duration of the playback (tape time), the time elapsed until completion of blood collection (handling time), and the time from the start of the playback until completion of blood collection (total time). After blood collection, we banded each vireo with a U.S. Fish and Wildlife aluminum band and a unique combination of color bands. Birds were also marked with white correction fluid (Liquid Paper) on crown feathers for sex identification during incubation observations.

## Behavior

Within 2–5 days of blood collection, we observed the behaviors of the parents at the nest for at least 1 h during the incubation and/or feeding stages, between 08:00 and 16:00 EST, from a minimum distance of 10 m (Van Roo, in preparation). In addition to confirming the parental behaviors for both species reported by earlier accounts of unbanded birds, these observations allowed us to determine the current breeding stage of each individual.

## Hormone measurements

Upon capturing a bird, we used a 26-gauge needle and heparinized microhematocrit tubes to collect approximately 250  $\mu$ l of blood from a brachial vein. Blood was centrifuged and plasma was removed and frozen for PRL and/or T radioimmunoassay. T and PRL were measured from the same sample when sample volumes were large enough to allow this (60% of samples). Samples of smaller volume were

randomly assigned to either the T or PRL assay. Each individual was used for only a single data point, corresponding to its breeding stage at the time of blood collection. All procedures were in compliance with and authorized by the Animal Care and Use Committee of Indiana University.

Testosterone was measured as described by Wingfield and Farner (1975). Briefly, T was extracted from plasma with anhydrous diethyl ether, separated from other steroid hormones using column chromatography, and free hormone was separated from bound with dextran-coated charcoal. The mean recovery value for radiolabeled T was 45%. Testosterone antibody was from Wien Laboratories (Succasunna, NJ), T standard was from Sigma (St. Louis, MO), and radiolabeled T was from New England Nuclear (Boston, MA). The lowest detectable value on the standard curve was 1.95 pg/tube. Samples below the detectable range were examined by two methods: They were either (1) assigned a value of zero or (2) designated as the mean scintillation count for the lowest detectable standard (1.95 pg/tube). Both methods provided similar statistical results; statistics reported here were produced by the latter method. A separate assay was performed for samples from 1997 and 1998 and those from 1999. The intraassay coefficients of variation were 16 and 12%, respectively, and the interassay coefficient of variation was 16%.

Plasma PRL was measured using a radioimmunoassay for recombinant-derived starling (*Sturnus vulgaris*) PRL as described by Bentley et al. (1997). The parallelism of the inhibition and standard curves showed good cross-reactivity of vireo PRL with recombinant-derived European starling PRL. The intraassay coefficient of variation was 6.8% and the interassay coefficient of variation was 10%.

### Statistical analyses

All data were examined for normality and equality of variances prior to analysis with parametric tests. For each hormone we first used a two-way analysis of variance (the factors were species and breeding stage) to determine whether the two species differed in overall hormone concentration and whether there was a species-by-breeding stage interaction indicating that hormone profile differed by species. Next, we examined each species in a separate one-way ANOVA to determine whether hormone level varied with breeding stage for that particular species. If the ANOVA was significant, we used Bonferroni multiple comparison post hoc tests to determine which stages differed (Sokal and Rohlf, 1998). Bonferroni multiple comparison post hoc tests (hereafter: post hoc) were also used to compare both species at particular stages. Finally, we used a one-way ANOVA to determine whether PRL levels varied with sex (females had no measurable T levels). Most samples from Blue-headed Vireo females were obtained during the incubation and nestling stages so we compared PRL levels in Blue-headed Vireo males and females at these stages only. Plasma samples from Red-eyed Vireo females

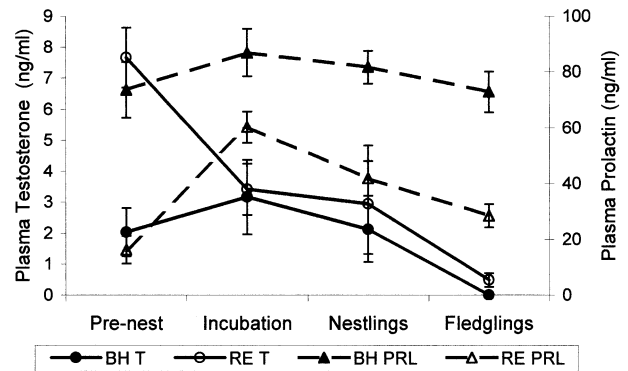


Fig. 1. Plasma hormone levels of male Blue-headed Vireos and male Red-eyed Vireos across reproductive stages (mean  $\pm$  SE). Sample sizes across reproductive stages for Blue-headed Vireo testosterone (9, 7, 10, 3) and prolactin (9, 8, 10, 3). Sample sizes for Red-eyed Vireo testosterone (14, 4, 9, 13) and prolactin (11, 5, 10, 7).

were few and unevenly distributed across stages so they were not included in any analyses. We used linear regressions to determine which covariates (year, day of year, time of day, tape time, handling time, total time) to include in the analyses. Only significant covariates are noted in the results. All analyses were performed using SPSS 10.0 software (1999).

## Results

### Plasma testosterone

Plasma T concentrations ranged between 0–9.0 and 0–17.6 ng/ml in male Blue-headed Vireos and Red-eyed Vireos, respectively.

There was a significant species-by-breeding stage interaction for plasma T concentrations [ $F(3, 68) = 3.07, P = 0.034$ ], therefore we analyzed each species separately with regard to variation across breeding stages. In male Blue-headed Vireos, plasma T did not vary with breeding stage but remained consistently low throughout the breeding season (Fig. 1). In contrast, plasma T in male Red-eyed Vireos declined significantly across breeding stages [ $F(3, 40) = 10.5, P < 0.001$ ]. Plasma T concentrations in male Red-eyed Vireos, which were high during the prenesting stage, had decreased significantly by the nestling stage [post hoc,  $F(1, 23) = 4.5, P = 0.024$ ].

Averaged across all breeding stages, mean plasma T concentrations were 2.49 ng/ml (SE = 0.43) and 4.00 ng/ml (SE = 0.70) in male Blue-headed Vireos and Red-eyed Vireos, respectively, and did not differ statistically [ $F(1, 68) = 2.8, P = 0.097$ ]. Plasma T concentrations were significantly higher in male Red-eyed Vireos than Blue-headed Vireos during the prenesting stage [post hoc,  $F(1, 22) = 17.0, P < 0.001$ ] and were not different at any other breeding stage (post hoc).

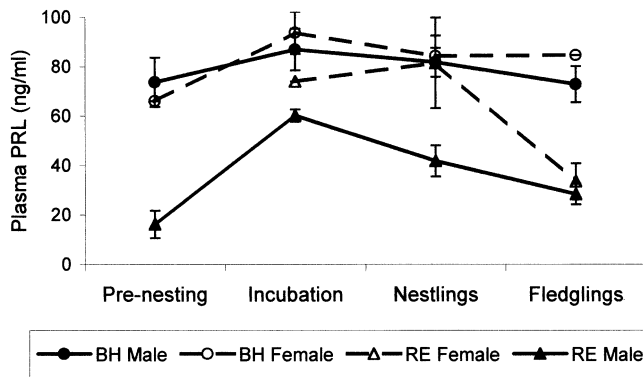


Fig. 2. Plasma prolactin levels of male and female Blue-headed Vireos and Red-eyed Vireos at different reproductive stages (mean  $\pm$  SE). Sample sizes across reproductive stages for Blue-headed Vireo males (9, 8, 10, 3) and females (1, 11, 9, 1). Sample sizes for Red-eyed Vireo males (11, 5, 10, 7) and females (0, 1, 2, 3).

### Plasma prolactin

Plasma PRL concentrations ranged between 26–136.5 and 0–90 ng/ml in male Blue-headed Vireos and Red-eyed Vireos, respectively. Averaged across all breeding stages, mean plasma PRL concentrations were 77.8 ng/ml (SE = 4.4) and 32.2 ng/ml (SE = 3.8) in male Blue-headed Vireos and Red-eyed Vireos, respectively. Because the two species differed significantly in overall plasma PRL concentrations [ $F(1, 63) = 76.3, P < 0.001$ ], we analyzed each species separately with regard to variation across breeding stages. Day length (rather than the day of year) covaried significantly with PRL concentrations in both male Blue-headed Vireos [ $F(1, 30) = 8.8, P = 0.002$ ] and Red-eyed Vireos [ $F(1, 33) = 17.6, P < 0.001$ ] and was included as a covariate in subsequent analyses. Tape time covaried significantly with PRL concentrations in male Red-eyed Vireos and was included in subsequent analyses [ $F(1, 33) = 19.7, P = 0.032$ ].

Breeding stage had no effect on plasma PRL in male Blue-headed Vireos (Fig. 2). In contrast, plasma PRL varied significantly with breeding stage in male Red-eyed Vireos [ $F(3, 35) = 4.5, P = 0.010$ ]. Plasma PRL concentrations in male Blue-headed Vireos were high throughout the breeding season, whereas in male Red-eyed Vireos, they were low during the prenesting stage and increased significantly by the incubation stage [post hoc,  $F(1, 21) = 25.7, P < 0.001$ ] and then decreased again by the fledgling stage [ $F(1, 12) = 31.8, P < 0.001$ ].

When we directly compared species at each breeding stage, plasma PRL concentrations were significantly higher in male Blue-headed Vireos than in Red-eyed Vireos at each breeding stage, although this difference was only marginally significant during incubation [prenesting  $F(1, 24) = 31.7, P < 0.001$ ; incubation  $F(1, 17) = 4.1, P = 0.059$ ; nestling  $F(1, 22) = 27.0, P < 0.001$ ; fledgling  $F(1, 11) = 38.4, P < 0.001$ ].

### Comparing plasma PRL across sexes

In male Blue-headed Vireos, mean plasma PRL concentrations did not differ from those of females at either the incubation stage ( $n = 8$  and 11, respectively) or the nestling stage ( $n = 10$  and 9, respectively, Fig. 2). No female Red-eyed Vireos were sampled during the prenesting stage and only one was sampled during incubation, so the sexes were compared using an ANOVA for PRL levels at the nestling stage and an ANOVA for PRL levels at the fledgling stage. Plasma PRL in female Red-eyed Vireos ( $n = 2$ ) was higher than in males ( $n = 10$ ) during the nestling stage [ $F(1, 10) = 5.8, P = 0.037$ ] but not the fledgling stage ( $n = 3$  and 7, respectively).

### Comparing plasma PRL in females

When females of both species were compared at the nestling stage, plasma PRL concentrations in female Red-eyed Vireos did not differ from those in female Blue-headed Vireos (84.2 ng/ml, SE = 7.9,  $n = 2$  and 81.5 ng/ml, SE = 16.7,  $n = 9$ , respectively). Although the power of this test was very low due to small sample sizes for females, the similarity between the means suggests that the species did not differ.

## Discussion

On average, males of both species did not differ in the overall magnitude of the plasma T concentrations whereas male Blue-headed Vireos, the more parental species, had higher overall plasma PRL concentrations.

In addition to the magnitude of hormone concentrations, the patterns of hormone concentrations across breeding stages were compared between species as well. The two species differed in whether the pattern of hormone levels varied with breeding stage. Male Blue-headed Vireos, with their extensive paternal care, maintained low T and high PRL across all breeding stages in contrast to male Red-eyed Vireos that demonstrated a more typical pattern of an early peak in T that declined with a simultaneous increase in PRL (Ball, 1991).

### Testosterone

In male Red-eyed Vireos, plasma T exhibited the high peak during prenesting stage that is typical of north temperate songbirds (Wingfield et al., 1990). The prenesting peak in male Red-eyed Vireos was significantly higher than plasma T during later breeding stages in this species and significantly higher than prenesting plasma T in male Blue-headed Vireos. The relatively low plasma T concentrations in Blue-headed Vireo males early in the breeding season are unusual and unlike the Red-eyed Vireo. Although high concentrations of T are not necessary for territory establish-

ment (Wingfield, 1994) or successful copulation (Moore and Kranz, 1983), a point exemplified by tropical species (Wingfield et al., 1991; Wikelski, Hau, and Wingfield, 1998), an early season peak in T was found in other species with male incubation, including male Semipalmated Sandpipers, with biparental incubation, and in several sex-role reversed species (Gratto-Trevor et al., 1990a; Oring et al., 1988; Fivizzani and Oring, 1986). Apparently, male Blue-headed Vireos establish and maintain territories without elevating T during territory formation, much like tropical species (see Hau et al., 2000; Hau et al., 2001).

### *Prolactin*

Like most birds that demonstrate some form of care, plasma PRL in male Red-eyed Vireos was low early in the breeding season and increased during the incubation stage, although still remaining lower than that of male Blue-headed Vireos. In contrast, plasma PRL in male Blue-headed Vireos was high early in the breeding season and remained high throughout. High PRL early in the season may be functionally important to Blue-headed Vireos in promoting brood patch formation. During the day, male Blue-headed Vireos incubate as much as females and exhibit feather loss, vascularization, and edema of the abdomen similar to that of females, although to a lesser extent (James, 1973; personal observation). Brood patches may develop in male Blue-headed Vireos as a result of the actions of PRL (Hutchison et al., 1967) or of both PRL and T (Johns and Pfeiffer, 1963). Initiation of brood patch development might therefore explain the high PRL concentrations in male Blue-headed Vireos during the prenesting stage.

Elevated PRL in male Red-eyed Vireos during the incubation stage, despite the lack of incubation behavior, is consistent with observations in males of other species (Campbell et al., 1981; Dawson and Goldsmith, 1982; Silverin and Goldsmith, 1983; Hector and Goldsmith, 1985; Dufty and Wingfield, 1986; Hiatt et al., 1987; Wingfield et al., 1990). The increase in plasma PRL in nonincubating males during the incubation stage of the breeding cycle could be a response to increased photoperiod (Ebling et al., 1982; Silverin and Goldsmith, 1997; Sharp et al., 1998; Hiatt et al., 1987) and/or to the visual stimulus of incubating females (Silver et al., 1973; Cheng, 1975). The functional significance of the increase in plasma prolactin in nonincubating male Red-eyed Vireos may be related to a behavior seen in other Red-eyed Vireo populations in which males feed their mates on the nest, allowing females to continue incubating without leaving the nest to forage (Southern, 1958). Feeding mates on the nest could be considered an indirect form of parental care, and PRL may facilitate this feeding behavior in the same manner it is believed to promote the feeding of young (e.g., Brown and Vleck, 1998). However, we have only observed this behavior once in our Red-eyed Vireo population, so its association with elevated

PRL levels in Red-eyed Vireo males during the incubation stage remains speculative.

The lack of a difference in plasma PRL concentrations between male and female Blue-headed Vireos is consistent with observations in other species in which males and females contribute equally to incubation (Myers et al., 1989; Gratto-Trevor et al., 1990b; Seiler et al., 1992; Williams and Sharp, 1993). We were unable to determine whether female Red-eyed Vireos also demonstrated higher plasma PRL than males during the incubation stage, as seen in other species in which one sex dominates incubation (Goldsmith, 1982; Ketterson et al., 1990; Schoech et al., 1996; Oring et al., 1986; Gratto-Trevor et al., 1990b). However, female Red-eyed Vireos maintained higher plasma PRL than males during the nestling period, when females were feeding young at a greater rate than males. Similarly, sex differences were found in plasma PRL concentrations of Red-necked Phalaropes, with strictly male incubation, but not seen in Semipalmated Sandpipers, in which the sexes share incubation (Gratto-Trevor et al., 1990a, 1990b).

Previous comparisons of hormone profiles of males with different modes of care have involved diverse groups of unrelated birds, e.g., shorebirds, penguins, albatrosses, songbirds, and domesticated species. Until now, a comparison of closely related species that differ in the presence or absence of a particular paternal behavior has not been made. The results provide a valuable comparison that fits well with our current understanding of the roles of these hormones and the differences in breeding behaviors of the two species.

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