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Regulation of Yolk-Androgen Concentrations by Plasma Prolactin in the American Kestrel

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The concentrations of maternally derived androgens in the yolks of avian eggs vary within and among clutches, but a mechanistic basis for this variation has not been elucidated. We investigated in the American kestrel, *Falco sparverius,* whether changes in plasma-prolactin concentrations induced by changes in photoperiod and food supply affect yolk-androgen concentrations. Over the course of a photoinduced breeding period in the laboratory, we measured concentrations of plasma immunoreactive prolactin (ir-prolactin) in female kestrels with *ad libitum* food availability (control) or food availability that was reduced during the early breeding period. In a second laboratory study, we administered via osmotic mini-pumps ovine prolactin (o-prolactin) to females beginning on the day they laid their first egg of a clutch (egg-day 1) to determine the effects of high prolactin concentrations on yolk-androgen concentrations. In both this study and one on free-living kestrels, we quantified changes in yolk-androgen concentrations with date of clutch initiation. Concentrations of ir-prolactin in nonlaying females rose with date, irrespective of food treatment. Egg-day 1 ir-prolactin concentrations were higher in control females laying late during the breeding phase than in those laying early. This increase was absent in food-reduced females. Yolk-androgen concentrations in eggs 3 and 4 but not eggs 1 and 2 of the clutch were higher in clutches initiated late than in clutches initiated early in the breeding phase in both the field and laboratory. o-Prolactin treatment elevated yolk-testosterone but not androstenedione concentrations. These findings suggest that, in American kestrels, seasonal and laying-associated increases in plasma-prolactin concentrations elevate yolk-testosterone concentrations. Food availability and other factors may interact with date to regulate the effects of prolactin on yolk-testosterone deposition.

Key Words: **egg laying; *Falco sparverius;* maternal effects; optimal reproductive tactic; seasonal breeding; yolk testosterone.**

Vertebrate egg yolks contain maternally derived hormones (Dickhoff, Brown, Sullivan, and Bern, 1990; Janzen, Wilson, Tucker, and Ford, 1998; Lipar, Ketterson, and Nolan, 1999a). Concentrations of yolk androgens vary with environmental conditions and may influence reproductive success (Schwabl, 1996a, 1996b, 1997; Schwabl, Mock, and Gieg, 1997; Gil, Graves, Hazon, and Wells, 1999; Bowden, Ewert, and Nelson, 2000; Sockman and Schwabl, 2000). In birds, ovarian follicles grow over 2–3 weeks from a diameter of 1 mm to a full-sized ovum at ovulation (Johnson, 1996), and the period of rapid yolk deposition occurs during the approximately 10 days preceding ovulation (Romanoff and Romanoff, 1949; Astheimer, 1985; Meijer, Masman, and Daan, 1989; Etches, 1996). It is during this time of rapid yolk deposition, when the ovum is still contained in the follicle and surrounded by the steroidogenic theca and granulosa cells forming the follicular walls, that androgens are likely deposited in the yolk.

In canaries, *Serinus canaria,* and American kestrels, *Falco sparverius,* yolk androgens influence nestling growth rates and differ greatly in concentrations among sibling yolks (Schwabl, 1993, 1996b; Sockman and Schwabl, 2000). Differences in growth rates among nestlings may promote a competitive hierarchy among siblings, raising the possibility that the deposition of yolk androgens may serve as a mechanism for female birds to favor some offspring over others (Schwabl *et al.,* 1997). Yolk-testosterone (T) concentrations in canaries vary with date of clutch initiation, resulting not only in a seasonal decline in overall concentrations but also in the seasonal loss of within-

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clutch variation (Schwabl, 1996a) and possibly a seasonal change in maternal favoritism. Despite evidence in birds (Schwabl, 1996a; Lipar, Ketterson, Nolan, and Casto, 1999b) and reptiles (Bowden, Ewert, Lipar, and Nelson, 2001) that the maternal hormonal milieu may influence the deposition of yolk steroids, a mechanistic basis for the variable deposition of yolk androgens has not been elucidated.

The purpose of this study was to investigate the potential role of changes in plasma-prolactin concentrations in the regulation of seasonally changing yolk-androgen concentrations in American kestrels (i.e., change with date of clutch initiation). Prolactin is generally regarded in birds as antigonadotropic (Goldsmith, 1983; Zadworny, Shimada, Ishida, and Sato, 1989; Buntin, Advis, Ottinger, Lea, and Sharp, 1999), and plasma concentrations of prolactin increase rapidly during and after clutch formation (e.g., Etches, Garbutt, and Middleton, 1979; Sockman, Schwabl, and Sharp, 2000) and slowly over the course of the breeding season (e.g., Silverin and Goldsmith, 1997). Additionally, there is limited evidence suggesting that fasting (e.g., Millam and El Halawani, 1986) and low ambient temperature (Maney, Hahn, Schoech, Sharp, Morton, and Wingfield, 1999) may depress prolactin concentrations, whereas handling stress may increase them (El Halawani, Silsby, Fehrer, and Behnke, 1985). Changes in circulating prolactin concentrations are known to reduce follicular steroidogenesis in fowl (e.g., Zadworny *et al.*, 1989). Because the steroidogenic follicular cells (Bahr, Wang, Huang, and Calvo, 1983) are likely the sources of androgens in the yolk, prolactin concentrations during the phase of rapid yolk deposition of follicles may influence yolk-androgen concentrations.

The American kestrel is a socially monogamous falcon, which, in our free-living study population, may initiate clutches between late March and late June (K. W. Sockman and H. Schwabl, unpublished data). This relatively long breeding season facilitates the study of seasonal change in reproductive physiology and behavior. American kestrels typically lay one egg every 2 days, but laying intervals of 1 and 3 days sometimes occur. In the closely related European kestrel, *Falco tinnunculus*, rapid yolk deposition (and probably the deposition of yolk androgens) occurs over approximately 9 days (Meijer *et al.*, 1989), a period that is likely similar to that in the American kestrel. In the American kestrel, concentrations of yolk T and androstenedione (A_4) are lower in the first-laid than in the later laid eggs of a clutch, and experimentally elevated concentrations of yolk androgens delay hatching, reduce nestling growth and survival rates, and elevate concentrations of plasma corticosterone in nestlings (Sockman and Schwabl, 2000, 2001). We conducted field and laboratory studies to test the hypothesis that in the American kestrel, seasonal changes in female plasma-prolactin concentrations, induced by changes in photoperiod and food supply, contribute to seasonal variation in yolk-androgen concentrations. We predicted that (1) concentrations of yolk androgens would decline seasonally (i.e., with date of clutch initiation), as they do in the canary; (2) plasma-prolactin concentrations in females would rise seasonally, as has been shown in several bird species; and (3) exogenous prolactin administered to laying females would reduce concentrations of yolk androgens, due to an antigonadotropic action of prolactin.

MATERIALS AND METHODS

We adhered to the standards of the Washington State University Institutional Animal Care and Use Committee (in accordance with the National Institutes of Health) for the humane treatment of our subjects. Our general laboratory and field procedures were outlined previously (Sockman and Schwabl, 2000; Sockman *et al.*, 2000). Below we describe procedures specific to this study.

Laboratory Study I

In this study, we investigated changes in plasma-prolactin concentrations in females over the course of a photoinduced breeding period. We obtained American kestrels (all age 3.5 years) in December 1997 from McGill University, Quebec, Canada, where they had been held in outdoor aviaries on a naturally changing photoperiod. These birds were born and raised in captivity as were several generations of their progenitors. We randomly formed 18 pairs in individual pens within one room; held them on an 8-h light, 16-h dark photoperiod (8L:16D); provided them with water; and fed them three or four 1-day-old cockerel chicks per day (*ad libitum*). We equipped pens (approximately 0.7 m wide $\times$ 2.3 m high $\times$ 2.0 m deep) with perches and a nesting box (inside width $\times$ depth $\times$ height: 17.4 $\times$ 16.3 $\times$ 33.8 cm) and lined their floors with pine shavings. On January 24, 1998, we changed the photoperiod to 10L:14D; on January 31 to 12L:12D; and on February 7 to 14L:10D (similar to late April at the latitude of the field study).

Beginning with the change to 12L:12D, we randomly assigned nine pairs to a temporary reduced food availability of two cockerel chicks per day (early

food-reduced group). We maintained the other nine on *ad libitum* food availability (control group). We based this treatment on the observation that, on short photoperiods when pairs were not engaged in reproduction, pairs typically consumed two chicks per day. We assumed this amount would not satiate laying females, which, given the opportunity, would increase their food intake for egg production (e.g., Meijer *et al.*, 1989). We returned early food-reduced pairs to *ad libitum* food availability on April 3, 1998, a point 9 weeks after transfer to 12L:12D and after which all laying females had completed first clutches. This moderate level of early food reduction had no effect on male or female body mass, date of clutch initiation (days from transfer to 14L:10D to onset of laying), or clutch size (Sockman *et al.*, 2000).

We collected ca. 400-μl blood samples from brachial veins of females every 2 weeks, beginning immediately before the first increase in light hours and lasting through the 12 weeks that we maintained them on a 14L:10D photoperiod. To monitor photoinduced changes in prolactin concentrations that were not associated with laying, incubation, or care of the young (hereafter called basal concentrations), we excluded from analyses samples collected from females within (before or after) 10 days of having eggs or young. We also collected blood from females on the day they laid their first egg of a clutch (egg-day 1) to examine prolactin concentrations at a specific time early in the laying cycle. We stored blood on ice for a few hours before centrifugation at 9000 rpm for 9 min to separate plasma, which we stored at −20°C. We conducted this study concurrently with and using the same birds as one previously published (Sockman *et al.*, 2000).

Field Study

In this study, we investigated seasonal change (change with date of clutch initiation) in yolk-androgen concentrations in the eggs of females breeding in nest boxes we hung along roadsides within a 35-km radius of the Washington State University campus (Pullman, WA, 46°N44,′ 117°W10′). During spring of 1997 and 1998 we checked boxes every 3–4 days for signs of occupancy. We estimated date of clutch initiation using the modal laying interval of 2 days and the number of eggs in the nest when we first observed laying. Due to the variation in laying interval (see the introduction), we may have erred by 1 or 2 days in estimating date of clutch initiation in some nests (checking boxes too frequently causes pairs to abandon them). During laying, we checked boxes every 1–3 days and marked new eggs. We collected and froze within a few days of the eggs' being laid either yolk samples obtained by biopsy (see Schwabl, 1996a) or whole eggs. This latency from laying to yolk collection does not affect androgen concentrations (Sockman and Schwabl, 2000). Some females laid up to six eggs, but we have no yolk samples from egg 6 and only three from egg 5 of the laying sequence. The size of most clutches from which we collected yolk samples was four eggs. Therefore, we limited our analyses to the first four eggs in the laying sequence. We used data from renests of three females because we did not have data from their first nests.

Laboratory Study II

In this study, we examined whether exogenous prolactin administered to laying females affects yolk-androgen concentrations. In addition, to examine changes in yolk-androgen concentrations with date of clutch initiation, we compared yolk-androgen concentrations between multiple clutches (renests) of individual pairs. On September 18, 1999, we transferred 18 pairs from a photoperiod of 8L:16D to 10L:14D and stepped the photophase up to 14L:10D by 2 h/week (as in laboratory study I). Once laying had begun, we checked boxes daily and marked eggs as they were laid. We collected yolk biopsies from eggs within a few days of their being laid.

On the afternoon a particular female laid the first egg of her first clutch only, we subcutaneously implanted in the scapular region of that female an osmotic pump (Model No. 1007D: Alza Corporation, Palo Alto, CA) containing 100 μl of either vehicle (0.03 M $NaCO_3$, 0.15 M NaCl, pH 8.7) as a control or 5 μg ovine prolactin (NIDDK oPRL-21)/μl vehicle. We have shown ovine prolactin (o-prolactin) to be biologically active in kestrels (Sockman *et al.*, 2000). According to manufacturer specifications, pumps dispensed at a rate of approximately 0.56 μl/h. Thus, they dispensed for approximately 7.5 days. We randomly assigned o-prolactin or control treatment to females. Delivery rates in these pumps (calculated from concentrations and the pump rate) were 0 and 2.8 μg o-prolactin/h for each concentration. Immediately after inserting the pump, we sealed the incision with Nexaband surgical glue (Veterinary Products Laboratories, Phoenix, AZ). According to manufacturer specifications, the pumps require approximately 4 h before they reach maximal delivery rates. In an effort to elevate prolactin concentrations as quickly as possible, we augmented pumps with a single, subcutaneous 100-μl injection of either 0 (control) or 0.028 μg o-pro-

lactin/μl vehicle. We returned females to their pens within approximately 30 min of their removal.

Six days after clutch completion, we removed pumps and collected the eggs, stimulating renesting. Six days after completion of second clutches, we again removed eggs, stimulating some females to initiate third clutches. We administered o-prolactin only when females were laying their first clutches but collected yolk samples from all clutches.

Measurement of Yolk Androgens and Plasma Prolactin

We followed previously described procedures (Sockman and Schwabl, 2000) for the extraction, separation, partial purification, and measurement by radioimmunoassay of yolk T and A_4, with the exception that we omitted the hexane wash. The sensitivities of the yolk T and A_4 assays were 1.95 and 3.90 pg/tube, respectively. We measured yolk A_4 in one assay for the field study and one for the laboratory study. We measured yolk T in two assays for the field study and one for the laboratory study. We placed both a positive and a negative control at the end of each assay for the field study and evenly distributed each of three positive and three negative controls for each assay for the laboratory study. For each assay, we used nine serially diluted standards to form a standard curve. The A_4 interassay coefficient of variation was 7.2%, and intraassay coefficients of variation were 10.7% (field study) and 5.2% (laboratory study). The T interassay coefficient of variation was 19.0%, and intraassay coefficients of variation were 7.3% (field study, most samples), 4.1% (field study, remaining samples), and 3.1% (laboratory study). Although the T interassay coefficient of variation was somewhat high, the major source of variation was between the field assays and the laboratory assay; we do not compare laboratory and field values in any analyses. The interassay coefficient of variation for the field assays alone was 2.3%. Neither yolk-T nor A_4 concentrations in the field differed between 1997 and 1998 (Sockman and Schwabl, 2000).

Immunoreactive prolactin (ir-prolactin) was measured in duplicates of 25 μl of plasma diluted to a 100-μl volume in one radioimmunoassay, conducted in the laboratory of P.J.S., using antisera against recombinant-derived starling prolactin, and following the protocol of Bentley, Goldsmith, Dawson, Glennie, Talbot, and Sharp (1997). The intraassay coefficient of variation and sensitivity were, respectively, 8.1% and 101 pg/tube. Serial dilutions of pooled plasma samples paralleled standard dilutions, indicating that this heterologous radioimmunoassay can be used to assess relative concentrations of plasma ir-prolactin in American kestrels (see Sockman *et al.*, 2000, for statistics and serial dilution curves).

Statistical Analyses

For the laboratory studies, we used analyses of variance (type III sums of squares) for repeated measures to analyze change in basal ir-prolactin with respect to date (with food treatment as a factor) and to analyze yolk androgens among eggs within a clutch (with o-prolactin treatment as a factor). For laboratory study I, we used analysis of covariance to analyze the effects of date of clutch initiation (covariate) on egg-day 1 ir-prolactin (with food treatment as a factor). For laboratory study II, we hierarchically built into models renests as repeated measures to form a double repeated-measures analysis. That is, we repeatedly measured yolk androgens among eggs within each of the second and third clutches of individual females to determine the effects of clutch number on yolk-androgen concentrations. For the field study, we used simple linear regression to determine whether yolk-androgen concentrations changed with date of clutch initiation. Sample sizes for all tests are indicated in the figures.

RESULTS

Laboratory Study I

For each of the 18 females in this study, we collected blood samples and measured plasma ir-prolactin concentrations through the first 4 weeks following the onset of the photoinduced breeding phase (i.e. the change from 8L:16D to 10L:14D). Thereafter, sample sizes declined as females began laying. Basal concentrations of ir-prolactin rose significantly [$F(7, 43) = 18.63$, $P < 0.0001$] with date (Fig. 1A). Neither food treatment nor the interaction between food treatment and date affected basal ir-prolactin concentrations. The first significant rise in basal ir-prolactin occurred by 2 weeks after we had changed the photoperiod to 14L:10D, as revealed by post hoc linear contrasts. ir-Prolactin continued to rise until 8 weeks following transfer to 14L:10D, a point at which concentrations began to reach a plateau (Fig. 1A). Sample sizes for the second half of the breeding phase were small because most females during that period were within 10 days of having either eggs or nestlings (see Methods).

Food treatment did not affect egg-day 1 ir-prolactin

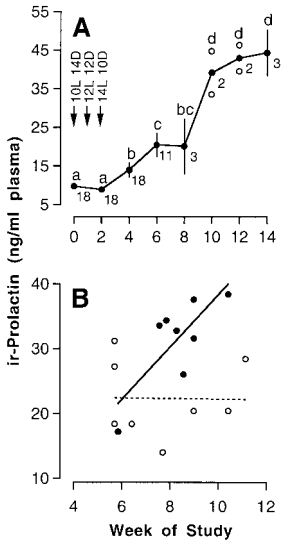

FIG. 1. Rise in plasma immunoreactive prolactin with date in laboratory-housed female American kestrels. Both (A) basal concentrations of ir-prolactin (means ± 1 SEM) using only those plasma samples collected from females not within 10 days of having eggs or nestlings and (B) concentrations on the day egg 1 of a clutch was laid (egg-day 1) were measured. For egg-day 1, data for both control (solid symbol and solid line) and early food-reduced (open symbol and dotted line) females are shown. These groups were pooled for basal concentrations. Kestrels were maintained on a photoperiod of 8L:16D before onset of the study (week 0). Blood samples were collected immediately before females experienced the changes in photoperiod, indicated by the arrows. Thus, week 0 hormone concentrations reflect those of birds on an 8L:16D photoperiod. Points with at least one letter in common were statistically indistinguishable ($P \geq 0.05$) based on post hoc linear contrasts. Numbers beside each point indicate the number of females used at each point. Because data were available for only two females at weeks 10 and 12 (A), the mean is plotted with the raw data (open symbols) rather than the SEM.

concentrations. However, date [$F(1, 12) = 4.57$, $P = 0.054$] and the interaction between food treatment and date [$F(1, 12) = 4.77$, $P = 0.050$] each affected egg-day 1 ir-prolactin concentrations. For 2 of the 16 females that laid in this study, we did not have measures of egg-day 1 ir-prolactin for their first clutches of the breeding phase. Therefore, we used data from their second clutches (after eggs from their first clutches were removed to induce renesting). If we exclude these two females from the analysis, the interaction between food treatment and date is still significant [$F(1, 10) = 5.90$, $P = 0.036$], but the main effects of date and food treatment are not.

To better understand the significant relationship between egg-day 1 ir-prolactin concentrations and the interaction factor described above, we regressed egg-day 1 ir-prolactin on date of clutch initiation separately for each of the two food treatments (Fig. 1B). In control females [$F(1, 6) = 9.04$, $P = 0.024$, $r^2 = 0.61$], but not females with early food reduction, there was a significant positive correlation.

Field Study

Females from whose eggs we had yolk samples initiated clutches over a period of 78 days (from April 8–June 25). Yolk-A_4 concentrations were substantially higher than yolk-T concentrations, and variation in both was considerable among eggs 1–4 of the laying sequence (Fig. 2). We detected no change with respect

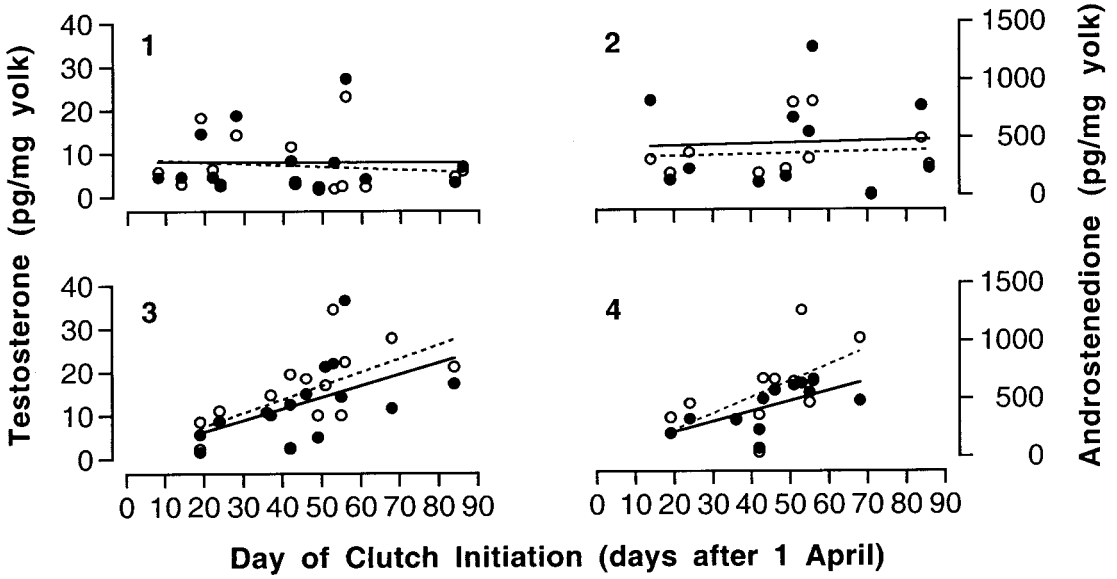

FIG. 2. Seasonal change in yolk-androgen concentrations in the eggs of free-living American kestrels. Concentrations of yolk testosterone (solid symbols and solid line) and androstenedione (open symbols and dotted line) were measured in eggs 1–4 of the laying sequence of a clutch.

to date of clutch initiation in yolk T or A_4 in both eggs 1 and 2 of the laying sequence. Both T and A_4 in eggs 3 [T: $F(1, 13) = 4.92$, $P = 0.045$, $r^2 = 0.27$; A_4: $F(1, 13) = 8.03$, $P = 0.014$, $r^2 = 0.38$] and 4 [T: $F(1, 10) = 6.58$, $P = 0.028$, $r^2 = 0.40$; A_4: $F(1, 10) = 5.24$, $P = 0.045$, $r^2 = 0.35$] of the laying sequence were positively correlated with date of clutch initiation (Fig. 2).

Laboratory Study II

By the time we began administration of exogenous o-prolactin, females had laid egg 1, and ovum 2, having likely been ovulated (Porter and Wiemeyer, 1972; Porter, 1975), was probably no longer accumulating androgens. We expected o-prolactin to affect only follicles still in the phase of rapid yolk deposition at the time o-prolactin administration began. Therefore, we selected eggs 3 and 4 *a priori* for statistical comparison of yolk androgens between treatment groups.

In eggs 3 and 4 of the laying sequence, yolk T [$F(1, 14 = 6.06$, $P = 0.03$], but not A_4, was significantly higher in females treated with o-prolactin than in control females (Figs. 3A and 3B). As expected, for eggs 1 and 2, treatment groups did not differ in either yolk T or A_4 concentrations (Figs. 3A and 3B). Because some females laid egg 2 more than 2 days after egg 1, it is possible that these females had not yet ovulated ovum 2 by the time prolactin administration began and that yolk 2 was still accumulating T. This may explain why T concentrations in egg 2 showed a trend similar to that in eggs 3 and 4 (Fig. 3A). In an analysis of control females only, concentrations of yolk T [$F(3, 20) = 6.61$, $P = 0.0028$] and A_4 [$F(3, 20) = 7.84$, $P = 0.0012$] were significantly lower in egg 1 than in eggs laid subsequently in the laying sequence of the clutch (Figs. 3A and 3B). We did not analyze the effect of laying sequence on yolk androgens for females treated with o-prolactin due to the effect of o-prolactin on yolk-androgen concentrations and the fact that o-prolactin treatment began after some yolks had been ovulated.

Administration of o-prolactin may have delayed ovulation and hence increased the time available to yolks for accumulation of T, causing yolk T concentrations to rise. To investigate this possibility, we analyzed the effects of o-prolactin treatment on the interval between laying of eggs 3 and 4 (that interval most likely to be affected by o-prolactin treatment) and found there was no treatment effect. Likewise, there was no treatment effect when we analyzed the laying intervals between eggs 1 and 2, 2 and 3, and 3 and 4 together in a repeated-measures analysis of variance.

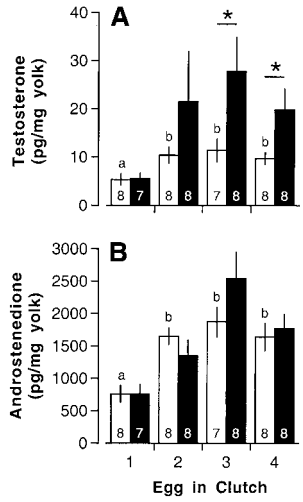

FIG. 3. Effect of laying order and ovine prolactin administered to laying, laboratory-housed, female American kestrels on yolk-androgen concentrations (mean ± 1 SEM). Vehicle (open bars) or ovine prolactin in vehicle (solid bars) was administered to females on the day they laid their first egg of a clutch, and yolk concentrations of (A) testosterone and (B) androstenedione were measured in eggs 1–4 of the laying sequence of a clutch. Asterisks indicate where differences between treatments were significant ($P < 0.05$) based on post hoc linear contrasts. For females receiving vehicle only, bars with a letter in common were statistically indistinguishable ($P \geq 0.05$) based on post hoc linear contrasts. Numbers at the base of each bar indicate the number of eggs for each bar.

Females in the second laboratory study initiated laying in first clutches over a 31-day period. There was no effect of date of clutch initiation on yolk-T or A_4 concentrations for any of eggs 1–4, possibly because the period of first-clutch initiation was less than half that in the field or possibly because the effects of o-prolactin overwhelmed effects induced by date (recall that o-prolactin was administered only for first clutches). However, we collected yolk samples (eggs 1–4) from four females that laid second and third clutches. This allowed us to look for change in yolk androgens with date by comparing second with third clutches, long after the pumps had dispensed their contents and been removed. We combined in this analysis females previously treated with either o-prolactin ($n = 2$) or vehicle ($n = 2$). There were no overall differences in androgen concentrations between yolks from clutches 2 and 3. However, there was a significant egg × nest interaction for both T [$F(3, 9) = 6.69$, $P = 0.01$] and A_4 [$F(3, 9) = 11.48$, $P = 0.002$], indicating that yolk-androgen concentrations in some eggs increased between clutches but did not in other eggs (Fig. 4). Post hoc linear contrasts

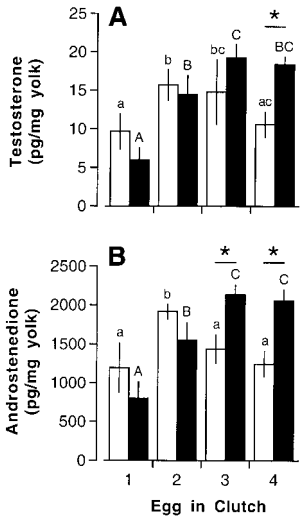

FIG. 4. Yolk-androgen concentrations (mean ± 1 SEM) in sequential clutches (renests) in laboratory-housed American kestrels. Yolk concentrations of (A) testosterone and (B) androstenedione were measured in second (open bars) and third (solid bars) clutches for eggs 1–4 in the laying sequence of a clutch. Asterisks indicate where differences between clutches 2 and 3 were significant ($P < 0.05$) based on post hoc linear contrasts. Within a clutch, bars with at least one letter in common were statistically indistinguishable ($P \geq 0.05$) based on post hoc linear contrasts. Each bar has a sample size of four eggs.

indicated that yolk T increased between clutches 2 and 3 for egg 4 and that yolk A_4 increased between clutches 2 and 3 for eggs 3 and 4.

We also examined the relationship between laying order within a clutch and yolk-androgen concentrations as we did for clutch 1 of control females (see above). There was a significant laying order effect for both T [$F(3, 9) = 9.35$, $P = 0.004$] and A_4 [$F(3, 9) = 6.13$, $P = 0.015$]. For clutch 3, the relationship between laying order and yolk-androgen concentrations was similar to that in clutch 1, with significantly lower concentrations of both T and A_4 in egg 1 than in subsequently laid eggs (Figs. 4A and 4B). However, for clutch 2, concentrations of yolk T were low in egg 1, relatively high in eggs 2 and 3, and then low again in egg 4. Yolk A_4 was low in egg 1, relatively high in egg 2, and then relatively low in eggs 3 and 4.

DISCUSSION

In females with *ad libitum* access to food, basal and egg-day 1 concentrations of plasma ir-prolactin rose with date. Yolk androgens increased with date of clutch initiation in eggs 3 and 4 of the laying sequence. Exogenous o-prolactin administered to laying females resulted in an increase in yolk-T concentrations. Together these results support the hypothesis that seasonally rising concentrations of plasma prolactin contribute to a seasonal increase in yolk-T concentrations in eggs 3 and 4 of the laying sequence.

Rise in Prolactin with Date

Our observation that basal concentrations of ir-prolactin rose with date in laboratory birds (Fig. 1A) is consistent with studies on other species (see the introduction for citations), including the European kestrel (Meijer, Daan, and Hall, 1990). This rise with date also occurred for samples collected from control females on egg-day 1 (Fig. 1B), a time when the rate of yolk deposition in eggs 3 and 4 would have been high. This correlation suggests that changes in plasma ir-prolactin during the breeding season may contribute to seasonal changes in concentrations of yolk androgens. The principle driver for this increase in prolactin is long day length (Sharp, Dawson, and Lea, 1998), but other environmental factors may also be involved, including food supply and other environmental stressors (see the introduction). Our results indicate that one of these environmental factors—mild, early food-reduction—may inhibit the seasonal rise in prolactin. Food-induced modifications in the relationship between prolactin and date may enable females to adjust yolk-androgen deposition in late-laid eggs of a clutch such that androgen concentrations are appropriate for a particular ecological environment. Other environmental factors, such as ambient temperature, inclement weather, social stressors, and more severe food reduction, should be examined more thoroughly in the specific context of the role ir-prolactin plays in the regulation of yolk-androgen concentrations.

Changes in Yolk Androgens with Date of Clutch Initiation and Maternal Prolactin Concentrations

In contrast to canaries (Schwabl, 1996a), yolk androgens in kestrels did not decline but, in fact, increased with date of clutch initiation in eggs 3 and 4 of the laying sequence (Figs. 2 and 4). Our findings that exogenous prolactin elevated yolk-T concentrations (Fig. 3) were surprising because we predicted that an antigonadotropic action of prolactin (see the introduction) would, if anything, reduce yolk-T concentrations. Nonetheless, our findings are consistent with the hypothesis that seasonal changes in prolactin secretion contribute to seasonal changes in patterns of yolk-androgen deposition.

The within-clutch pattern of androgen concentra-

tions in clutches 1 and 3 of the laboratory study was similar to that previously reported for the American kestrel (Sockman and Schwabl, 2000); the canary (Schwabl, 1993); and the red-winged blackbird, *Agelaius phoeniceus* (Lipar *et al.*, 1999a). That is, concentrations of yolk androgens were low in the first-laid egg of a clutch and higher in subsequently laid eggs. It has previously been shown that plasma-prolactin concentrations in female kestrels and other species rapidly increase over the course of laying (Sockman *et al.*, 2000). Because plasma prolactin in laying females seems to elevate concentrations of yolk androgens, these findings suggest that, in addition to its possible role in seasonally changing yolk-androgen concentrations, prolactin may also be important in regulating the within-clutch increase in yolk-androgen concentrations that occurs in some species (but see Schwabl *et al.*, 1997; Gil *et al.*, 1999, for alternative within-clutch patterns of yolk-androgen deposition).

Surprisingly, yolk androgens did not show this within-clutch pattern of deposition for clutch 2 of the laboratory study. Although concentrations in egg 1 were low, they were also low in egg 4. The reason for this inconsistency is not clear but may be related to renesting shortly after exogenous o-prolactin treatment. For example, administration of exogenous prolactin may have temporarily down-regulated prolactin receptors mediating the effect of prolactin on deposition of yolk androgen. Patterns of androgen deposition in relation to laying sequence may have important fitness consequences (Schwabl, 1996b; Sockman and Schwabl, 2000) and should be investigated in unmanipulated renesting females and under a variety of ecological conditions to determine the extent to which within-clutch patterns of androgen deposition vary.

Yolk-T concentrations in egg 4 and A_4 concentrations in eggs 3 and 4 increased from second to third clutches of individual females (Fig. 4). Although this increase may have been influenced by the earlier prolactin treatment and is not completely analogous to the seasonal change in yolk androgens we observed in the field, it may suggest that similar processes occurred under laboratory and field conditions. Moreover, concentrations of yolk androgens and plasma prolactin were similar between the laboratory and field, suggesting that the laboratory environment did not grossly alter these variables.

In canaries, concentrations of yolk A_4 are somewhat lower than T concentrations, and yolk A_4 is undetectable in the zebra finch, *Poephila guttata* (Schwabl, 1993). However, in the cattle egret, *Bubulcus ibis* (Schwabl *et al.*, 1997), and the American kestrel (Sockman and Schwabl, 2000; this study), concentrations of yolk A_4 are much higher than T concentrations. The functional significance and mechanistic basis of this difference in concentrations is not known.

The mechanism by which prolactin elevates yolk testosterone remains to be discovered as well. The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) irreversibly converts dehydroepiandrosterone to A_4. 17β-Hydroxysteroid dehydrogenase (17β-HSD) reversibly converts A_4 and T, and aromatase irreversibly converts A_4 to estrone and T to estradiol. It is possible that prolactin inhibits the activity of aromatase, as observed in the ovaries of rats (Dorrington and Gore-Langton, 1981; Tsai-Morris, Ghosh, Hirshfield, Wise, and Brodie, 1983; Papadopoulos, Drosdowsky, and Carreau, 1986) and enhances the activity of both 3β-HSD and 17β-HSD, as observed in the testis of the bonnet monkey, *Macaca radiata* (Gunasekar, Kumaran, and Govindarajulu, 1988). If ovarian A_4 is abundant and its conversion to T limited by 17β-HSD activity, as might be suggested by the much higher yolk concentrations of A_4 compared to T, then enhancement of 17β-HSD combined with inhibition of aromatase might lead to a net accumulation of T, possibly also within the yolk (Zelinski-Wooten, Hess, Baughman, Molskness, Wolf, and Stouffer, 1993). Replenishment of A_4 by prolactin-induced inhibition of aromatase and enhanced activity of 3β-HSD might lead to relatively little change in A_4, as we observed in the yolk.

Also speculative is the possibility that prolactin enhances testosterone secretion by the adrenal glands, which may then elevate testosterone concentrations in the yolk. Rats express adrenal receptors for prolactin (Calvo, Finocchiaro, Luthy, Charreau, Calandra, Engstrom, and Hansson, 1981), and hyperprolactinemea in women is associated with elevated plasma concentrations of testosterone (Glickman, Rosenfield, Bergenstal, and Helke, 1982). Whether these findings apply to birds is not known.

Our results support the hypothesis that a seasonal rise in prolactin stimulates a seasonal rise in yolk T. However, this study does not readily explain why yolk T rises seasonally in only eggs 3 and 4 but not in eggs 1 and 2. Although plasma-prolactin concentrations in female kestrels rise rapidly over the course of laying a clutch (Sockman *et al.*, 2000), they are at their lowest during the rapid phases of yolk deposition for follicles 1 and 2 of a clutch. It is possible that, regardless of date, concentrations of plasma prolactin at this time in the laying cycle are too low to enhance T deposition. However, as the female begins to spend more time incubating during clutch formation, the associated increase in plasma prolactin concentrations (Sockman *et al.*, 2000) may reach a threshold above

which the deposition of T into growing follicles increases. The consequence of the seasonal increase in plasma-prolactin concentrations might be that females laying near the end of the season reach this threshold earlier during the formation of clutches than females laying near the beginning of the season.

Clutch size in kestrels declines seasonally (Dijkstra, Vuursteen, Daan, and Masman, 1982; Sockman and Schwabl, 2001). We would expect females laying larger, earlier clutches to reach the prolactin threshold for elevated deposition of yolk T relatively late in laying, causing concentrations of yolk T in eggs 5 and 6 to be relatively high compared to those in eggs 3 and 4. The relationship between laying date and prolactin concentrations, combined with the effects of prolactin on yolk T, suggests a mechanism by which females might ensure high concentrations of yolk T in eggs laid late in the sequence of a clutch, regardless of clutch size and date. Additional studies on concentrations of yolk androgens in all eggs of varying clutch sizes are necessary. To better support the finding that prolactin up-regulates yolk T deposition, additional studies involving experimentally reduced prolactin might prove useful as well. For example, we would predict that female kestrels immunized against prolactin or the prolactin releasing factor vasoactive intestinal polypeptide (Dawson and Sharp, 1998) would deposit relatively little testosterone into the yolks of their eggs.

It is certainly likely that factors other than circulating prolactin influence seasonal change in yolk-androgen deposition. In some species, such as the canary in which seasonal patterns are opposite those observed in kestrels, such putative factors may play a more important role than prolactin. We suggest prolactin as one of potentially several factors regulating the deposition of yolk androgens and thus regulating the hormonal control of sibling competition and offspring fitness.

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