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# The Role of Prolactin in the Regulation of Clutch Size and Onset of Incubation Behavior in the American Kestrel

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**In most bird species, the timing of incubation onset may influence the degree of hatching asynchrony, which, together with variation in clutch size, affects reproductive success. In some domesticated species that usually show no hatching asynchrony, plasma prolactin concentrations in females rise with the onset of incubation and the end of laying, and this rise enhances incubation behavior and may terminate laying. To investigate whether a rise in prolactin during laying is involved in the regulation of clutch size and incubation onset in a species with hatching asynchrony, we measured plasma concentrations of immunoreactive prolactin (ir-prolactin) in laying American kestrels, *Falco sparverius,* and quantified clutch size and incubation behavior. In a separate study, we administered one of three concentrations of ovine prolactin (o-prolactin) via osmotic pumps implanted in females when egg 2 of a clutch was laid. ir-Prolactin concentrations during laying were higher in small than in large clutches and increased in parallel with the development of incubation behavior. o-Prolactin treatment enhanced incubation behavior, but did not affect clutch size, possibly because the manipulation was performed after clutch size had already been determined. Consistent with studies on domesticated species that show synchronous hatching, our results indicate that rising prolactin during laying enhances the expression of incubation behavior in a species that shows hatching asynchrony. Further studies are necessary to determine whether the relationship between prolactin and clutch size in the American kestrel is one of causation or of mere association.** 

***Key Words: egg laying, Falco sparverius,* family planning, hatching asynchrony, optimal reproductive tactic, reproductive success.**

Most bird species hatch their eggs asynchronously, producing a developmental and competitive hierarchy among siblings (reviewed in Magrath, 1990). Compared to synchronous hatching, asynchronous hatching may reduce parental (Hussell, 1972) and nestling (Hahn, 1981) energy expenditure during feeding of the young and facilitate the ease with which young are culled if food resources become scarce (Lack, 1947; Mock and Forbes, 1995). The degree of hatching asynchrony is largely influenced by the timing of incubation onset (e.g., Mead and Morton, 1985). If incubation begins before clutch completion, eggs tend to hatch asynchronously, with early-laid eggs first exposed to development-inducing temperatures hatching first.

A parallel strategy in times of food scarcity is to limit clutch size, which, in addition to establishing the maximum number of offspring produced, influences sibling competition (e.g., Stoleson and Beissinger, 1997). Considering the impact of clutch size and hatching asynchrony on the fitness of parents and nestlings, surprisingly little research has been directed toward determining the neuroendocrine mechanisms for the regulation of clutch size (Klomp, 1970) and incubation onset (Stoleson and Beissinger, 1995; Beissinger and Stoleson, 1997). Interestingly, these traits may be regulated by one and the same hormone (Eisner, 1960; Mead and Morton, 1985; Meijer, Daan, and Hall, 1990), prolactin.

The onset of incubation in species producing clutch sizes of more than two eggs is generally associated with increased plasma prolactin (Lea, Dods, Sharp, and Chadwick, 1981; Goldsmith, 1983; El Halawani, Fehhrer, Hargis, and Porter, 1988; Buntin, 1996; Sharp, 1997b). Immunization against prolactin or the prolac-

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tin-releasing-factor vasoactive intestinal peptide (Sharp, Dawson, and Lea, 1998) inhibits incubation (Crisóstomo, Guémené, Garreau-Mills, and Zadworny, 1997; Sharp, 1997a; Crisóstomo, Guémené, Garreau-Mills, Morvan, and Zadworny, 1998). Treatment of females with exogenous prolactin induces incubation if it is preceded by nesting behavior, which is dependent on increased plasma progesterone and estradiol 17-$\beta$ (El Halawani, Silsby, Behnke, and Fehrer, 1986; Sharp, 1997b). Thus, the transformation of nesting behavior to incubation behavior seems largely regulated by rising plasma prolactin levels during and immediately following laying (Lea *et al.*, 1981).

Some evidence suggests that plasma prolactin also plays a role in terminating egg laying and therefore in regulating clutch size in species laying clutches of more than two eggs. Cessation of egg laying is associated with increased concentrations of plasma prolactin (Etches, Garbutt, and Middleton, 1979; Burke and Dennison, 1980; Lea *et al.*, 1981; Bluhm, Phillips, and Burke, 1983; Hall and Goldsmith, 1983; Silverin and Goldsmith, 1983), and in turkeys (El Halawani, Silsby, Youngren, and Phillips, 1991) and domestic fowl (Sharp, Macnamee, Sterling, Lea, and Pedersen, 1988), administration of exogenous prolactin suppresses plasma gonadotropins necessary for egg production. However, there is additional evidence that the suppression of gonadotropin secretion in incubating birds also involves a mechanism independent of increased prolactin secretion (Sharp *et al.*, 1988; Lea and Sharp, 1989; Sharp, Sterling, Talbot, and Huskisson, 1989; Lea, Richard-Yris, and Sharp, 1996).

The rate or timing of incubation onset with respect to clutch completion (and therefore the degree of hatching asynchrony) varies interspecifically, intraspecifically, and intraindividually (Haftorn, 1981; Bortolotti and Wiebe, 1993; Schwabl, 1996; Wiebe, Wiehn, and Korpimäki, 1998; Sockman and Schwabl, 1999, unpublished). However, the evidence above that increased plasma prolactin is involved in the regulation of both the onset of incubation behavior and termination of egg laying comes from species that usually show no hatching asynchrony. Comparative studies on the role of prolactin in regulating clutch size and incubation onset are critical for elucidating these mechanisms that may have been shaped by the evolution of such a unique reproductive tactic as one employing hatching asynchrony. Therefore, we investigated the role of prolactin in the regulation of clutch size and incubation onset in the American kestrel, *Falco sparverius*, a species producing clutch sizes of four to six eggs and showing pronounced hatching asynchrony (Wiebe and Bortolotti, 1994). In free-living and laboratory kestrels, we measured changes in plasma prolactin during clutch formation and the development of incubation behavior and experimentally increased prolactin levels during egg laying, predicting that this manipulation would reduce clutch size and prematurely enhance incubation behavior.

## 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 animal subjects. We defined "egg-day" as the day on which a particular egg of a clutch was laid (see below for an amendment to this definition that applies to the field study only). For example, egg-days 1 and 3 were the days on which eggs 1 and 3 were laid. In kestrels, which usually lay every 2 days but sometimes every day or every 3 days, egg-day 3 in a female which laid every 2 days would not correspond to the same elapsed time after onset of laying as egg-day 3 in another which laid every 3 days.

### *Field Study*

We hung nest boxes (inside width $\times$ depth $\times$ height: 17.4 $\times$ 16.3 $\times$ 33.8 cm) at approximately 0.5-km intervals 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. Once laying had begun, we taped a thermistor to the inside of the nest-box floor and connected it through a small hole in the floor to a temperature logger (Onset Computer Corp., Pocasset, MA) positioned on a well-shaded shelf open on one side 3 cm below the floor of the box. We placed a second logger on this shelf to record the nest's ambient temperature. Loggers recorded temperature at intervals of approximately 3–5 min. We defined incubation as any time nest temperature was more than 6C° above ambient temperature, which reflected minimum temperature differences after clutch completion under most ambient temperatures (see Sockman and Schwabl, 1998 for a depiction of nest versus ambient temperatures). We calculated the percentage day (beginning and ending at 06:00) incubating for each day to quantify female incubation behavior on a daily basis. From field ob-

servations, the probability of seeing a female (versus a male or neither sex) in a nest box increased from 0.16 ± 0.09 (mean ± *SE*) during early laying, when incubation is nearly absent, to 0.87 ± 0.09 in midlaying to 0.91 ± 0.09 during late laying, when full incubation is nearly underway (ANOVA for repeated measures: $F_{(2,\ 23)} = 21.90$, $P < 0.0001$). This indicates that, even though free-living males sometimes incubate (Bortolotti and Wiebe, 1993), incubation temperatures during laying are strongly associated with the presence of a female in the box and are hence a measure of female behavior.

During laying, we checked boxes every 1–3 days and marked new eggs. Consequently, a particular "egg-day" could correspond either to the day that particular egg was laid or the day after. For example, egg-day 3 was the day, during laying, during which there were 3 eggs in the nest. (This does not apply to the laboratory studies, in which we checked boxes twice daily.) At various times during daylight hours, we captured adults in nest boxes or with a bal-chatri trap (Berger and Mueller, 1959), banded them, and collected blood samples of 400–600 μl from brachial veins. 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.

### Laboratory Studies

We obtained American kestrels (all age 3.5 years) in December 1997 from McGill University, Quebec, Canada, where they had been held 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 and held them on an 8-h light / 16-h dark photoperiod (8L 16D) and provided pairs with water and 3- to 4-day-old cockerel chicks per day (*ad libitum*). Pens were approximately 0.67 m wide × 2.34 m high × 2 m deep and equipped with perches and a nest box (as in the field). We determined incubation as in the field study, with the exception that a single logger recorded room temperature, which we compared to nest temperature to detect incubation. During laying, males rarely entered nest boxes, so temperature records reflect changes in female incubation behavior.

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 restricted diet of two chicks / day in an effort to experimentally delay laying and reduce clutch size. We maintained the other nine on *ad libitum* food. Beginning April 3, 1998, we returned restricted pairs to *ad libitum* food.

Beginning February 20, 1998, we weighed all birds at least once per week, except incubating females. Once laying began, we checked boxes at approximately 3 and 7 h after lights came on, marked eggs as they were laid, and collected approximately 400 μl of blood from females once they had laid eggs 1, 3, 4, 5, and 6 (hereafter on egg-days 1, 3, etc.). Therefore, we collected blood within a few hours of oviposition but at different times during the day (depending on whether the new egg was present on the first or second daily nest check). We also collected blood samples 5 or 6 days after the last egg of a clutch was laid. We stored and centrifuged samples as in the field study. Food restriction had no effect on male or female body mass, clutch size, or onset of laying. We therefore pooled these treatment groups for analyses and do not discuss this variable further. At least 6 days after clutch completion, we removed eggs from some nests, causing some females to renest.

To induce a second breeding period, we changed the photoperiod to 8L 16D on June 4, 1998. On October 9, 1998, we changed the photoperiod to 10L 14D and stepped it by 2-h intervals each week to 14L 10D. On egg-day 2, we subcutaneously implanted in the scapular region of each female (without anesthesia) an osmotic pump (model 1002: Alza Corporation, Palo Alto, CA) containing ovine (as opposed to avian) prolactin (NIDDK oPRL-21) because previous studies have shown the readily available ovine prolactin (o-prolactin) to be effective in birds (Pedersen, 1989; Youngren, El Halawani, Silsby, and Phillips, 1991) and to bind specifically to crude membrane fractions of ring dove, *Streptopelia risoria*, brain homogenates (Buntin and Ruzycki, 1987). Each pump contained 100 μl of one of three o-prolactin concentrations and pumped at a rate of 0.28 μl / h. Thus, they dispensed for approximately 15 days. The three o-prolactin concentrations were 0, 0.5, and 5 μg o-prolactin / μl vehicle (0.03 M $NaCO_3$, 0.15 M NaCl, pH ~8.6). Delivery rates in these pumps (calculated from concentrations and the pump rate) were 0, 2.3, and 23 ng o-prolactin / min for each concentration. Assuming a plasma volume of 7.5 ml (5% female body mass) and using an equation for the sum of an infinite geometric series (Judith D. Sockman, personal communication), we calculated the plasma concentration of o-prolactin at equilibrium between degradation and infusion of o-prolactin:

$$s = d / (1 - r) / v,$$

where $d$ is the delivery rate in nanograms per minute, $r$ is a term for a pituitary hormone with a half-life of 17 min (P. J. Sharp, unpublished data; $r = 0.5^{1/17}$), and $v$ is the circulating plasma volume. The calculated plasma concentrations were approximately 0, 8, and 80 ng o-prolactin / ml for each of the three o-prolactin concentrations administered. After inserting the pump, we sealed the incision with Nexaband surgical glue (Veterinary Products Laboratories, Phoenix, AZ) and returned the female to her pen within approximately 30 min of her removal. Immediately before pump implantation and again 4 days after pump implantation, we collected 400 $\mu$l of blood, which was stored and centrifuged as described above.

### Measurement of Immunoreactive Prolactin

ir-Prolactin was measured in duplicates of 25 $\mu$l plasma diluted to a 100-$\mu$l volume in two radioimmunoassays (interassay coefficient of variation = 11%). The assays were 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 for each assay were, respectively, 8.1% and 101 pg / tube for assay 1 (field and first laboratory breeding period) and 4.5% and 109 pg / tube for assay 2 (second laboratory breeding period). Serial dilutions of pooled samples from incubating and nonincubating birds paralleled standard dilutions (test for homogeneity of slopes: $F_{(2,\ 17)} = 0.18$, $P > 0.20$), indicating that this heterologous radioimmunoassay can be used to assess relative levels of plasma ir-prolactin in American kestrels (Fig. 1). o-Prolactin (NIDDK oPRL-18) did not displace the starling standard (Fig. 1), indicating that o-prolactin in plasma samples does not interfere with measurements of ir-prolactin.

### Statistical Analyses

For the laboratory studies, we used analyses of variance (type III sums of squares) for repeated measures, in which we quantified percentage day incubating or ir-prolactin among egg-days within nests. In the first laboratory study, we hierarchically built into models renests as repeated measures to form a double repeated-measures analysis. That is, we repeatedly measured ir-prolactin or incubation among egg-days within each of the first and renests. We used logistic regression to determine whether o-prolactin treatment affected clutch size. For the field data, we used simple

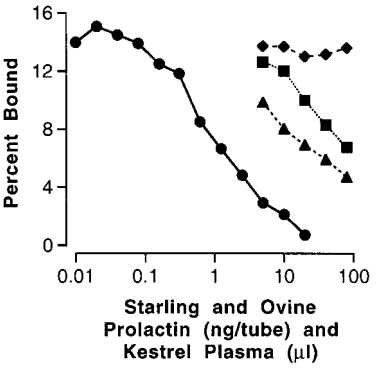


**FIG. 1.** Displacement curves of serially diluted American kestrel plasma pools (triangles: incubating females; squares: nonincubating males and females) and starling (circles) and ovine (diamonds) prolactin standard. Slopes did not differ among the curves for the starling prolactin and two plasma pools (see text). Because ovine prolactin did not displace the starling standard, this heterologous radioimmunoassay can be used to assess relative levels of plasma prolactin in American kestrels even when plasma samples contain ovine prolactin.

one-way analyses of variance because we had too few serially collected data for repeated-measures analyses. When multiple values for a single free-living female were available, we used only one in statistical analyses, selecting those samples necessary to distribute as evenly as possible the sample sizes among egg-days.

## RESULTS

In the first laboratory breeding period, 16 of 18 females laid complete clutches. Of these, 5 laid six-egg, 9 laid five-egg, and 2 laid four-egg first clutches. Ten females renested following egg removal, 3 laying six-egg, 4 laying five-egg, and 3 laying four-egg clutches. In the second laboratory breeding period, 18 of 19 females laid complete clutches (1 female died after clutch completion and was replaced with a new female). Of these, 16 had laid in the first breeding period, 5 laid six-egg, 10 laid five-egg, and 3 laid four-egg first clutches. Renests were not studied in the second breeding period.

In the field study, we analyzed plasma ir-prolactin on egg-days 1 and 2 in seven females, egg-days 3 and 4 in seven females, and egg-days 5 and 6 in six females. Because we installed temperature loggers at various times during laying, we analyzed incubation behavior on egg-days 1 and 2 in five females, on egg-days 3 and 4 in seven females, and egg-days 5 and 6 in five females. Free-living kestrels often abandoned

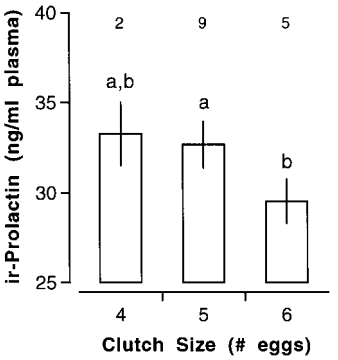


**FIG. 2.** Relationship between immunoreactive prolactin (mean ± 1 *SE*) and clutch size in laboratory American kestrels. ir-Prolactin was measured in (and averaged over) plasma samples collected on the days eggs 1, 3, and 4 were laid. Bars without at least one letter in common were significantly different ($P < 0.05$) based on post hoc Fisher's protected least significant difference. Numbers above the bars indicate the number of females in each clutch-size category.

nests if captured early during laying (i.e., egg-days 1–3). Consequently, we had few early-laying blood samples from females who continued to occupy boxes until clutch completion, and we were unable to analyze the relationship between clutch size and ir-prolactin levels during laying in the field study.

### Prolactin and Clutch Size

When we averaged (i.e., using ANOVA for repeated measures) ir-prolactin levels over egg-days 1, 3, and 4 only (because some females did not lay more than four eggs), ir-prolactin concentrations were significantly ($F_{(2,\ 12)} = 4.56$, $P = 0.034$) higher in females laying small than in females laying large clutches (Fig. 2). When the two four-egg clutches were removed from the model, the difference between five- and six-egg clutches remained significant ($F_{(1,\ 11)} = 7.77$, $P = 0.018$). Because we used renests in this analysis, we excluded a single female whose clutch size changed between her first and renest. The clutch sizes of the others' renests were the same as those of their first nests. o-Prolactin treatment did not affect clutch size ($\chi^2 = 6.29$, $df = 4$, $P = 0.18$; Table 1).

### Prolactin and Incubation Behavior

Incubation behavior and ir-prolactin levels increased in parallel over the course of laying in both the laboratory and the field (Figs. 3A and B). These increases were statistically significant in both the laboratory (incubation: $F_{(4,\ 47)} = 18.50$, $P < 0.0001$; ir-prolactin: $F_{(4,\ 46)} = 8.15$, $P < 0.0001$) and field studies (incubation: $F_{(2,\ 14)} = 34.44$, $P < 0.0001$; ir-prolactin: $F_{(2,\ 17)} = 4.12$, $P = 0.03$). ir-Prolactin was high 5–6

**TABLE 1**

Effects of Ovine Prolactin Treatment on Frequency of Clutch Sizes in the American Kestrel

| | o-Prolactin treatment (ng/min) | | |
|---|---|---|---|
| Clutch size | 0 | 2.3 | 23 |
| Four eggs | 2 | 0 | 1 |
| Five eggs | 4 | 1 | 5 |
| Six eggs | 1 | 3 | 1 |

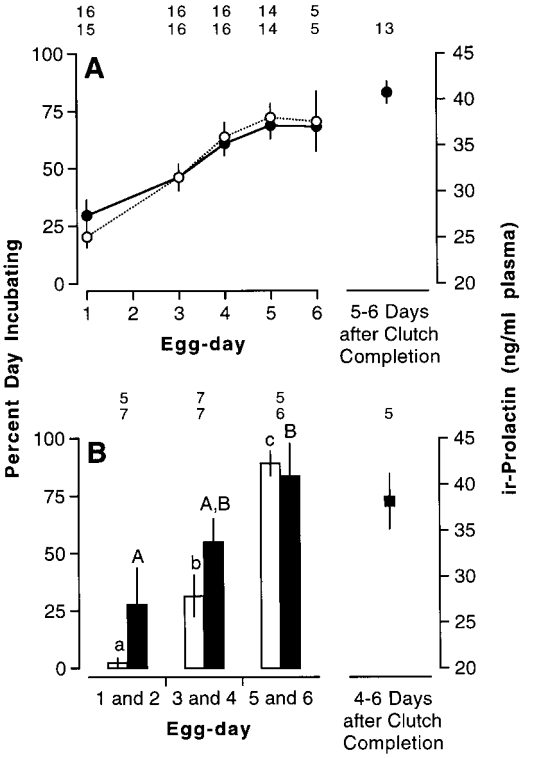


**FIG. 3.** Rise in incubation behavior and immunoreactive prolactin levels during laying in American kestrels. We measured incubation (means ± 1 *SE;* open symbols and bars) and ir-prolactin (means ± 1 *SE;* solid symbols and bars) according to day on which an egg was laid (egg-day) in (A) laboratory and (B) free-living American kestrels. The graphs also show ir-prolactin levels during full incubation several days after clutch completion. Points without at least one letter in common were significantly different ($P < 0.05$) based on post hoc linear contrasts. Due to the hierarchical nature of the repeated measures analysis in which egg-day was nested within nest number, linear contrasts were not estimable for the laboratory study. Numbers at the top of each graph indicate the number of females in which we measured incubation (top row) and ir-prolactin (bottom row) on a particular egg-day.

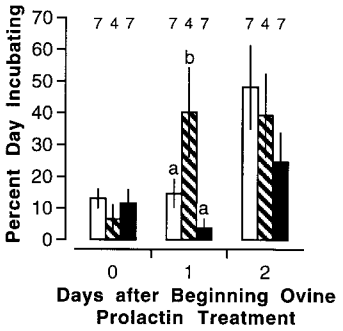


**FIG. 4.** Effect of ovine prolactin treatment (0 ng/min = open bars, 2.3 ng/min = hatched bars, 23 ng/min = solid bars) on incubation behavior in the American kestrel (means ± 1 *SE*). o-Prolactin was administered via osmotic pump implanted on the day the second egg of a clutch was laid. Points without at least one letter in common were significantly different ($P < 0.05$) based on post hoc linear contrasts. Numbers above the bars indicate the number of females in each treatment group.

and 4–6 days after clutch completion in the laboratory and field, respectively. Patterns of change and absolute levels of both incubation and ir-prolactin were similar between the laboratory and the field, indicating that captivity did not grossly alter either of these variables.

On the day pumps were implanted (egg-day 2) in the second laboratory study, incubation was low and did not differ significantly ($F_{(2, 15)} = 0.62$, $P > 0.2$) among the three treatment levels (Fig. 4). By 1 day after pump implantation, the 2.3 ng/min o-prolactin treatment but not the 23 ng/min o-prolactin treatment caused a significant increase in incubation over controls ($F_{(2, 15)} = 7.36$, $P = 0.006$). We did not detect significant differences in incubation with respect to treatment level for subsequent days.

### *Effect of o-Prolactin on ir-Prolactin*

To determine whether administration of o-prolactin affected levels of ir-prolactin, for example, through feedback inhibition, we subtracted ir-prolactin levels on egg-day 2 (i.e., those from blood samples collected immediately before pump implantation) from those 4 days later. The difference was positive for females receiving 0 ng o-prolactin/min (Fig. 5), reflecting the natural rise in ir-prolactin occurring over the course of egg laying (see Fig. 3). ir-Prolactin in females receiving 2.3 ng o-prolactin/min virtually did not change over this time period. The difference in ir-prolactin in females receiving 23 ng o-prolactin/min was negative and significantly different from controls ($F_{(2, 14)} = 3.81$, $P = 0.048$), indicating that o-prolactin suppressed ir-prolactin levels by 4 days after pump implantation.

## DISCUSSION

### *Prolactin and Clutch Size*

ir-Prolactin levels during laying were lower in large than in small clutch sizes (Fig. 2). To our knowledge, this is the first demonstration of a relationship between clutch size and ir-prolactin levels in laying females, although a previous study has shown such a relationship in postlaying females whose clutch sizes were manipulated with egg-addition and egg-removal experiments (Millam, Zhang, and El Halawani, 1996). Differences between four- and either five- or six-egg clutches were not significant, but this is probably because our sample size of four-egg clutches was only two females.

Egg-removal and egg-addition experiments in the zebra finch *Taeniopygia guttata* by Haywood (1993b) indicate that the tactile stimulation from one egg present in the nest is sufficient to disrupt follicular growth and therefore regulate clutch size. In the blue tit *Parus caeruleus* (Haywood, 1993a) and European kestrel *Falco tinnunculus* (Beukeboom, Dijkstra, Daan, and Meijer, 1988), the time (from clutch initiation) at which eggs in the nest disrupt follicular growth is positively correlated with clutch size. Porter (1975) found similar results in American kestrels, suggesting

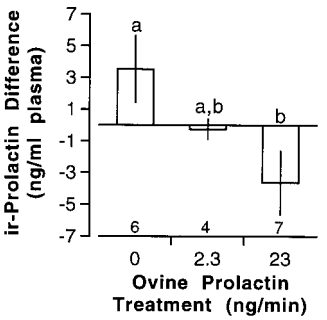


**FIG. 5.** Effect of ovine prolactin treatment on immunoreactive prolactin. We removed interfemale differences in ir-prolactin levels by determining the difference (means ± 1 *SE*) between levels 4 days after and levels immediately before the beginning of o-prolactin administration. A positive value indicates ir-prolactin increased over this time period. Bars without at least one letter in common were significantly different ($P < 0.05$) based on post hoc linear contrasts. Numbers below the bars indicate the number of females in each treatment group.

that females producing small clutches are more sensitive to stimuli from eggs in the nest and therefore respond earlier than females producing large clutches.

Do such differences in sensitivity giving rise to variation in clutch size translate into higher prolactin levels in females laying smaller clutches and is prolactin, therefore, the hormone causing follicular disruption in the American kestrel? It is possible, but our evidence is only correlative. Our prolactin manipulations on egg-day 2 failed to affect clutch size (Table 1), perhaps because the treatment started too late in the laying cycle. The experiments on blue tits and kestrels suggest that endogenous levels of prolactin may reach a threshold necessary for follicular disruption by egg-day 2 except in females laying large clutches. Therefore, it seems our administration of o-prolactin occurred too late in the laying cycle to affect clutch size.

The interpretation that rising plasma prolactin during laying regulates clutch size assumes that an antigonadotropic action of increased plasma prolactin is a major factor controlling the cessation of egg laying. This assumption may be incorrect because unidentified factors other than increased plasma prolactin are known to depress gonadotropin secretion in incubating birds (see the introduction for citations). The failure of exogenous prolactin to terminate laying is consistent with this view but belies the inverse relationship between clutch size and plasma ir-prolactin during laying of the initial four eggs of a clutch. Given these equivocal findings, whether prolactin plays a causal role in the regulation of clutch size in kestrels awaits further studies in which exogenous prolactin is administered earlier in laying and studies examining the role of other factors associated with high levels of prolactin.

### *Prolactin and Incubation Behavior*

The high levels of ir-prolactin during full incubation relative to levels early during laying and the positive relationship between ir-prolactin and the development of incubation behavior during laying (Fig. 3) are consistent with the hypothesis that increased plasma prolactin concentrations accelerate the onset of incubation behavior (see the introduction for citations). Increased expression of incubation behavior by administration of o-prolactin at a rate of 2.3 ng / min (Fig. 4) demonstrated that the relationship between prolactin and incubation onset is causal or permissive.

Surprisingly, administration of o-prolactin at a dose of 23 ng / min did not increase expression of incubation behavior, although it depressed endogenous concentrations of ir-prolactin (Fig. 5). This "short loop" feedback inhibition of prolactin secretion confirms observations in the rat (Nicholson, Greley, Humm, Youngblood, and Kizer, 1980) and turkey (El Halawani *et al.*, 1991). Our calculations (see "Materials and Methods") suggest that the administration of o-prolactin at 2.3 and 23 ng / min would have raised circulating concentrations by approximately 8 and 80 ng / ml plasma, respectively. As shown in Fig. 3A, concentrations of endogenous ir-prolactin increase by about 10 ng / ml plasma over the course of the development of incubation behavior. Administration of o-prolactin at 2.3 ng / min would therefore result in plasma concentrations within the physiological range. Administration at the higher level would result in circulating levels well above the physiological range and may have down-regulated brain prolactin receptors mediating the effect of prolactin on the expression of incubation behavior (Zhou, Zadworny, Guémené, and Kühnlein, 1996; Ohkubo, Tanaka, Nakashima, Talbot, and Sharp, 1998).

### *Summary*

Findings from this study are consistent with the view that in the American kestrel, increasing concentrations of plasma prolactin after the onset of laying enhance the expression of incubation behavior. As a consequence, embryonic development of early laid eggs in the clutch may begin before clutch completion, resulting in asynchronous hatching. The inverse relationship between female plasma prolactin during clutch formation and clutch size suggests that clutch size may be determined by the rate of increase in plasma prolactin during the early stages of laying. However, the failure of exogenous prolactin to reduce clutch size suggests that increasing concentrations of prolactin may not be the principal factor controlling clutch size.

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