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Daily Estradiol and Progesterone Levels Relative to Laying and Onset of Incubation in Canaries

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Female birds may optimize reproduction by modifying clutch size and the timing for the onset of incubation. We measured fecal estradiol-17β (E) and progesterone (P) in laying canaries to better understand how onset of incubation might regulate clutch size. Both E and P rose sharply to maxima 1 day before the first egg was laid. Thereafter, E steadily declined, but P remained high through 2 days after the first egg was laid, after which both hormones had returned to low levels. Clutch size did not explain variation in E or P output during the laying cycle. When analyzed with respect to onset of incubation, E and, to a lesser extent, P dropped significantly on the day incubation began, irrespective of whether or not females had finished ovulating. We suggest that factors initiating incubation also cause the decline in E production by small follicles, which in turn may inhibit yolk sequestration in large follicles. Further experiments in which onset of incubation is manipulated may reveal the mechanisms by which this behavior regulates clutch size and reproductive output.

Key Words: fecal steroid; clutch size; ovulation; *Serinus canaria.*

Female birds may adjust their reproductive output to the prevailing environmental conditions by modifying clutch size (Lack, 1954) and by establishing developmental hierarchies of young through asynchronous onset of incubation (Stenning, 1996). Lack proposed that changing or unpredictable food availability is the ultimate cause of plasticity in these traits, but the proximate causes of variation in clutch size and timing for incubation onset are largely unknown (Klomp, 1970; Magrath, 1990).

The regulation of clutch size and incubation onset may be linked by the putative dual role of the hormone prolactin. Some lines of evidence suggest that prolactin is anti-ovarian (Bates *et al.*, 1935; Meier, 1969) and that rises in circulating prolactin or the prolactin releasing factor vasoactive intestinal peptide (El Halawani *et al.*, 1997) may regulate clutch size (Youngren *et al.*, 1991; El Halawani *et al.*, 1995). Although we did not study prolactin directly, a consequence of rising prolactin may be the inhibition of ovarian steroid production. Determining daily changes in estradiol-17β and progesterone during laying may elucidate how clutch size is modified.

The functional significance of clutch size regulation is to determine the number of offspring produced. The functional significance of incubation onset is more obscure. Laying frequency does not exceed one egg per day, and, in many species, incubation begins prior to clutch completion (Haftorn, 1981; Mead and Morton, 1985; Beissinger and Waltman, 1991; Bortolotti and Wiebe, 1993; Meijer and Siemers, 1993; Wiebe *et al.*, 1998). Because first-laid eggs are exposed to development-inducing temperatures before later-laid eggs, first-laid eggs typically hatch first (but see Bortolotti and Wiebe, 1993; Viñuela, 1997), resulting in hatching asynchrony. Hatching asynchrony may produce developmental hierarchies among nestlings (Mead and Mor-

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ton, 1985; Evans, 1996; Nilsson and Svensson, 1996; Stoleson and Beissinger, 1997) and facilitate brood reduction (see Magrath, 1990). Female birds may vary the timing for the onset of incubation (Haftorn, 1981; Bortolotti and Wiebe, 1993; Schwabl, 1996; Wiebe *et al.*, 1998), enabling them to modify the developmental hierarchy of offspring and the probability of brood reduction according to variation in environmental factors such as food availability (Magrath, 1989; Wiebe and Bortolotti, 1994).

In addition to having anti-gonadal effects, a rise in prolactin stimulates onset of incubation in domesticated, precocial species (Riddle *et al.*, 1935; Lea *et al.*, 1981, 1982; Bluhm *et al.*, 1983; Hall and Goldsmith, 1983; El Halawani *et al.*, 1986; Youngren *et al.*, 1991) and possibly in the altricial canary (*Serinus canaria*; Goldsmith, 1982) and other taxa (Etches *et al.*, 1979; Goldsmith, 1983, 1990; Gratto-Trevor *et al.*, 1990), excluding Columbiformes (Lea, 1987). This dual role of prolactin raises the possibility that variation in clutch size and the establishment of developmental hierarchies are both consequences of onset of incubation.

We attempted to induce variation in clutch size and timing for incubation onset by manipulating photoperiod and food availability, two factors suggested by earlier work to affect female reproductive output (e.g., Lack, 1954; Schwabl, 1996). In an effort to better understand the hormones involved in regulation of clutch size and onset of incubation, we measured estradiol-17β (E) and progesterone (P) in fecal samples collected daily from laying canaries. We were particularly interested in how these steroids varied with onset of laying, clutch size, clutch completion, and onset of incubation.

METHODS

Experiment and Sampling Design

In a randomized complete split-plot design, we assigned 20 pairs of canaries to two rooms under a 12-h light/12-h dark photoperiod and to two rooms under a 16-h light/8-h dark photoperiod (5 pairs per room). Within each photoperiod, pairs were assigned to one of two food treatments (5 pairs per treatment per photoperiod). Control pairs were fed *ad libitum*, and food-restricted pairs were deprived of food during the middle 4 h of their photoperiod. Pairs were provided with water *ad libitum* and were visually but not acoustically isolated. Cages (32 cm high × 57 cm wide × 27 cm deep) were equipped with plastic nest cups and burlap strings for nest material. Lights in all rooms came on at 06:00. Rooms were held at approximately 20°.

We monitored incubation rhythms by placing into each nest cup a thermistor connected to a digital temperature logger (Onset Computer Corp., Pocasset, MA) that recorded nest temperature every 3.2 min. Although the exact locations of thermistors varied slightly among nests, they generally protruded up through the bottom of the nest and lay among the eggs. We defined incubation temperature as 4° above the maximum room temperature, measured by thermistors in nest cups of nonlaying and nonincubating females. Although nest temperatures during incubation were usually much higher, this definition allowed us to monitor the behavior of females whose thermistors had been shifted and were not otherwise directly beneath them. We calculated the percentage of the day during which the female maintained incubation temperature and defined the day on which 50% first occurred as the onset of incubation. For most nests, this method revealed an abrupt increase in incubation behavior during or shortly after laying (e.g., Fig. 1).

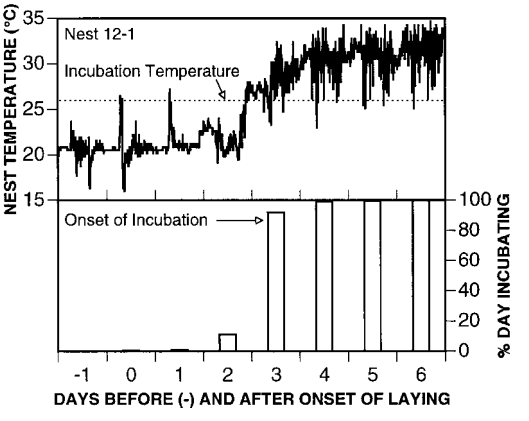

FIG. 1. Nest temperature and percentage of the day incubating, together showing timing for the onset of incubation in a single canary nest. Incubation temperature was defined as 4° above the maximum room temperature. After calculating the percentage day at incubation temperature, we defined the day on which 50% first occurred as the onset of incubation.

Although onset of incubation was gradual in some nests, we feel that variation in incubation behavior was discrete rather than continuous and that our definition of incubation onset was minimally arbitrary.

We collected fecal samples from all birds beginning with the day after the first female began laying and continuing daily for approximately 9 weeks. In addition, we collected samples from four laying females (three food-restricted and one control, all under the 16-L/8-D photoperiod) every 2 h during the photophase for 4 days.

Collection of all samples proceeded as follows: Beginning at 06:30 each morning, wire partitions were inserted to separate members of a pair, and the cage floors were lined with clean paper. A sample was usually collected within ½ h of changing the paper, after which the wire partition was removed. We rotated among the rooms in which we started changing papers and collecting samples. All daily samples were usually collected by 07:30, 90 min into the photophase. Within 1 h of collection, samples were placed in an oven at 25° until dry (usually 4–8 h), weighed, and frozen at −20° until they were prepared to assay.

Hormone Assays

We used two methods to assay for E and P. In method 1, we followed the procedure of Bishop and Hall (1991) for measuring the concentration of "free," unconjugated steroid in the feces. In this procedure, steroids are extracted in a phosphate buffer (60 µl buffer/mg feces). The E antiserum we used cross-reacts 100% with estradiol-17β, 14% with estrone, and 5% with estriol. The antiserum we used for P cross-reacts 100% with progesterone, 30% with deoxycorticosterone, 6% with 17-OH-progesterone, 5% with testosterone, 4% with androstenedione, and 4% with estradiol (both Arnel Products Co., New York, NY). Although the antisera probably cross-react with other steroids as well, immunoreactive E and P are hereafter referred to as E and P.

Validation of Hormone Assays

To determine the specificity of the method-1 assays, fecal samples from each of two laying females were subjected to method 2, described as follows: 40 µl of each fecal buffer extract were hydrolyzed with a 10-µl mixture of β-glucuronidase and sulfatase from *Helix pomatia* (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 18 h following the protocol of Payne and Talahay (1986). To determine the percentage steroid recovery, we then added 2000 cpm of ^{3}H-labeled P and E to each sample, vortexed them briefly, and let them equilibrate for at least 2 h. Steroids were then extracted with 2 × 4 ml of diethylether using Extrelut (EM Science, Gibbstown, NJ) minicolumns. Extracts were dried and steroids were separated and partially purified on diatomaceous earth chromatographic columns as described by Schwabl (1992, 1993) for blood samples. Samples were then assayed for E and P using radioimmunoassays.

For E, results from methods 1 and 2 were highly correlated for both females (Female 4: $F_{1,16} = 84.7$, $P < 0.001$, $r^2 = 0.84$; Female 12: $F_{1,18} = 118.7$, $P < 0.001$, $r^2 = 0.87$). The correlation for Female 4, whose mean E recovery was 78%, is shown in Fig. 2. Although hydrolyzation of extracts resulted in about 10× higher E levels, we used method 1, because these measurements of "free" E were highly correlated with those of "total" (free + conjugated) E.

There was little correlation between P measurements obtained with methods 1 and 2. It is possible that hydrolysis and organic extraction produce com-

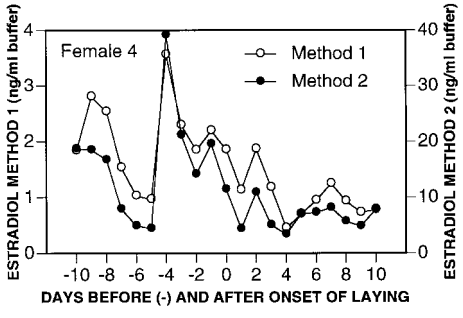

FIG. 2. Correlation of estradiol determined from two assay methods. Method 1 involved the extraction of steroids from feces with phosphate buffer followed by radioimmunoassay. Method 2 involved the extraction of feces with phosphate buffer, hydrolysis of the extract, extraction with organic solvent, and chromatographic separation and purification of estradiol, followed by radioimmunoassay. Note that in this and subsequent figures, levels are expressed as ng steroid/ml buffer, because we do not know the efficiency of steroid extraction with phosphate buffer.

pounds that coelute with the progesterone chromatography fraction and interfere with the antibody. Therefore, we further tested the specificity of method 1 by injecting approximately 100 ng ^{14}C-labeled P (DuPont NEN, Boston, MA; sp. act. = 55 Ci/mol) into the jugular veins of two nonlaying, photostimulated females and collected fecal samples every 30 min from the time that samples were first excreted (Samuel Wasser, pers. commun.). The approximate plasma volume of a canary is 1 ml, yielding an approximate P concentration of 100 ng/ml plasma shortly after injection. Although this is 10–20 times the plasma concentration for endogenous progesterone in laying females (Bluhm *et al.*, 1983; Seiler *et al.*, 1992; Yang *et al.*, 1997), it is probably within the physiological range. Injecting less P would have made it difficult to detect ^{14}C activity due to the relatively low specific activity. Fresh samples were weighed, and deionized water was added (1 µl water/mg fresh feces). Samples were briefly vortexed and ¼ of the total volume was transferred into a scintillation vial, to which an equal volume of ethanol was added. After adding 5 ml scintillation fluid, shaking, and overnight equilibration, samples were counted for ^{14}C (range of ^{14}C activity from fecal samples: 19–337 CPM). This allowed us to determine the excretion profile of P or its metabolites with which we could correlate a P profile obtained by a radioimmunoassay for the same samples. To this end, the remains of ^{14}C-labeled fecal samples were dried overnight at 45°. Samples were then extracted and assayed using method 1. Immunoreactive P correlated well with the excretion profile for both females (Female 1: $F_{1,16} = 33.1$, $P < 0.001$ $r^2 = 0.67$; Female 2: $F_{1,9} = 29.7$, $P < 0.001$ $r^2 = 0.77$). The results for Female 2 are shown in Fig. 3A. These results indicate that our antiserum binds P or its metabolites in proportion to the fecal excretion and probably blood levels.

To determine whether immunoreactive P levels of serially diluted fecal extracts paralleled standard dilutions, portions of 12 extracts showing high immunoreactive P levels were pooled. The pool was serially diluted six times and assayed for P in a single method-1 assay. Pool dilutions paralleled standard dilutions (Fig. 3B). Regression of log-transformed values of P concentration on the fraction bound revealed that the difference in the slope of pool dilutions and the slope of standard dilutions was not statistically significant

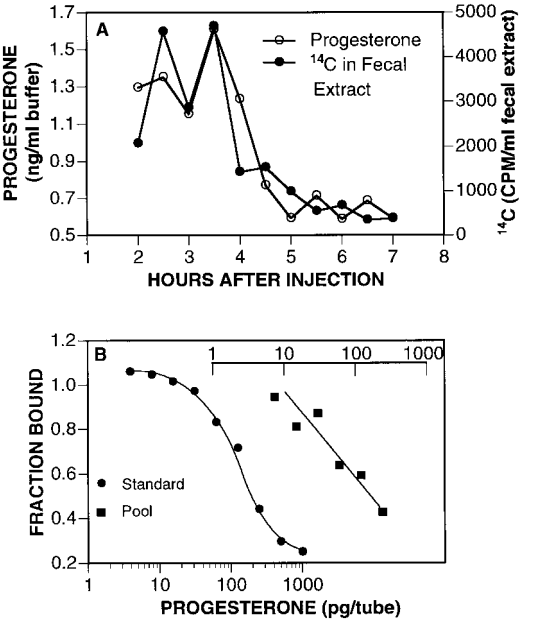

FIG. 3. (A) Correlation between immunoreactive progesterone as determined by radioimmunoassay in phosphate buffer extracts and ^{14}C-labeled fecal metabolite of progesterone after intrajugularly injecting ^{14}C-labeled progesterone into a female canary. (B) Displacement curves of serially diluted fecal samples from a pool of samples with high progesterone levels and serially diluted progesterone standard in the progesterone radioimmunoassay. Axis inset applies to pool dilution.

(slope ± 95% CI: pool = 0.33 ± 0.032; standard = 0.38 ± 0.036). Most of our P measurements fell onto the linear portion of the standard curve (40–80% binding).

We thus concluded that method 1 was specific for P and used it for all subsequent P assays.

Data Analyses

Timing for incubation onset can be measured with respect to initiation of laying (days after egg 1) or with respect to clutch completion (days before or after clutch completion). The effects of photoperiod and food regimen on clutch size and incubation onset were examined by analyses of variance for a randomized complete split-plot design with room as the experimental unit for the main factor and female as that for the split factor. Neither factor affected clutch size or incubation onset (measured either way), and we did not consider them further in subsequent analyses (see Results).

Because data collected repeatedly from individual sampling units (females) are inherently correlated, we used repeated measures analyses of variance to determine whether differences in E and P over time (day-to-day or bihourly) were statistically significant. After nests either failed or fledged young, some females renested. Nest number (renests) and day with respect to onset of laying were both factors built into a single repeated measures model, in which day was nested within nest number.

After conducting analyses of variance for repeated measures, we specified linear contrasts for post hoc comparisons of hormone levels before incubation began with levels after incubation began and levels before the last egg was laid with levels after the last egg was laid. Prior to data collection and analysis, we did not have specific interest in the numerous other possible comparisons, and therefore making contrasts for these would have increased the chance of making a type I statistical error.

We used simple one-way analysis of variance to determine whether differences in E and P with respect to clutch size were statistically significant. Figures show F and P values and means $\pm$ 1 SE.

RESULTS

Neither photoperiod nor food treatment significantly affected clutch size (photoperiod: $F_{1,2} = 0.27$, $P = 0.66$; food: $F_{1,14} = 0.78$, $P = 0.39$), onset of incubation with respect to initiation of laying (photoperiod: $F_{1,2} = 0.69$, $P = 0.49$; food: $F_{1,14} = 2.47$, $P = 0.14$), or onset of incubation with respect to clutch completion (photoperiod: $F_{1,2} = 0.47$, $P = 0.56$; food: $F_{1,14} = 2.84$, $P = 0.11$). Despite this, females showed substantial variation in clutch size and incubation onset (see below). This allowed us to look for correlations of these variables with steroid hormone levels, although no causal relationships could be assigned.

Fecal E levels showed substantial diel variation in four laying females (Fig. 4). E was significantly higher in samples collected 30 min into the photophase ($F_{7,18} = 11.34$, $P < 0.001$) than in samples collected later, but this relationship diminished with each subsequent day after onset of laying. By 4.5 h into the photophase or by 4 days after onset of laying, E was at low levels. Although E levels did not differ significantly among days 1–4 after onset of laying ($F_{3,8} = 3.213$, $P = 0.08$), there was a significant interaction between time of day and day after onset of laying ($F_{21,29} = 2.452$, $P = 0.01$).

Fecal P levels also showed statistically significant diel variation ($F_{7,18} = 9.002$, $P < 0.001$), but the pattern of change was less regular than that for E (Fig. 4). P did not change significantly either with respect to days after onset of laying ($F_{3,8} = 2.63$, $P = 0.12$) or with respect to the interaction between time of day and days after onset of laying ($F_{21,25} = 0.83$, $P > 0.2$). All subsequent analyses of E and P involved the first-collected samples in the photophase.

With respect to day-to-day variation, E and P levels rose from 10 days to a peak 1 day before onset of laying (Fig. 5). E levels then began a decline to reach low levels by 3 days after the onset of laying where they remained for at least 8 days. P levels, however, maintained a high plateau through 2 days after onset of laying. Thereafter, P levels dropped sharply to low levels where they remained for at least 8 days. The effect of nest number (renests) on E was not significant ($F_{2,7} = 4.27$, $P = 0.06$); however, P levels rose slightly with increasing nest number ($F_{2,6} = 6.96$, $P = 0.03$).

Clutch size ranged from one to four eggs, irrespective of photoperiod or food regimen. To determine whether the production of fecal E or P was related to clutch size, we summed E and P concentrations (from single samples collected at 06:30 each day) over those days bracketing laying during which Fig. 5 revealed high levels for the respective hormones. E concentrations were summed from samples collected 4 days before through 2 days after onset of laying and P concentrations from samples collected 1 day before through 2 days after onset of laying. Clutch size did not significantly explain variation in E or P production measured in this way (Fig. 6), although there was a tendency for higher levels of both hormones in females laying clutches of two to four eggs than in those laying one-egg clutches.

Females showed substantial variation in the onset of incubation relative to initiation of laying and to clutch completion. Again, this variation was irrespective of photoperiod or food regimen. Incubation began 4, 3, 2, and 0 days after initiation of laying (n = 2, 7, 9, and 2

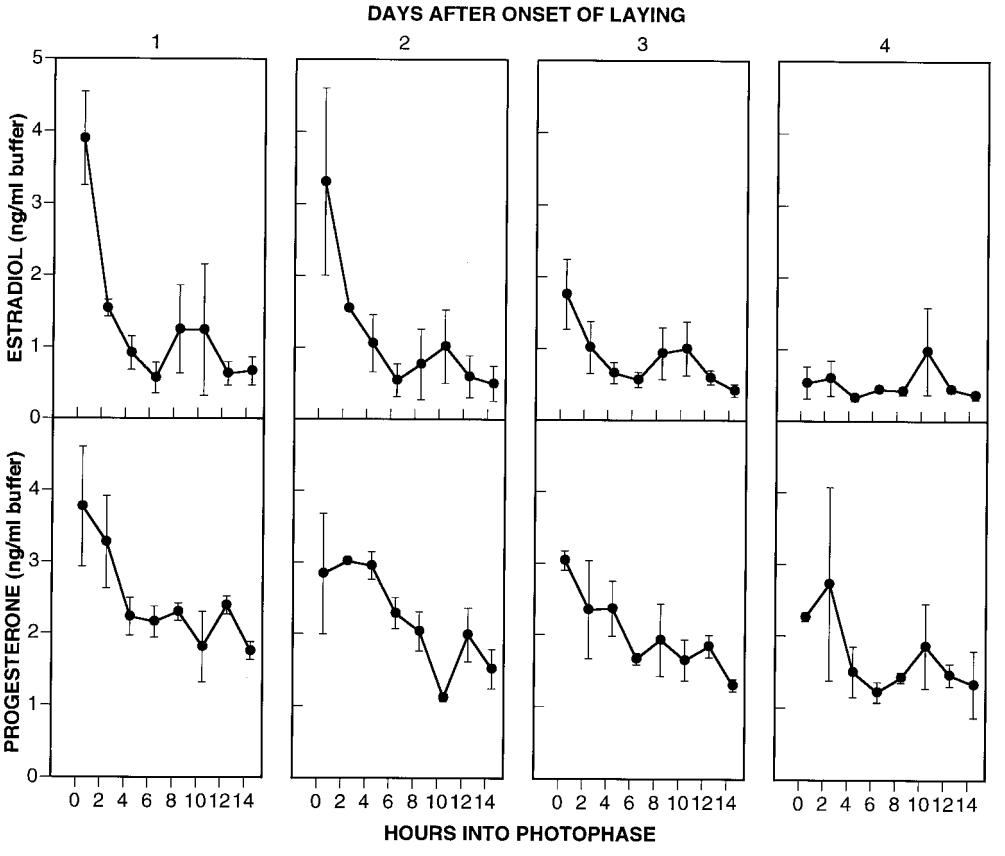

FIG. 4. Immunoreactive estradiol and progesterone in fecal samples collected bihourly from four female canaries during laying.

nests, respectively), or, alternatively, incubation began 4, 2, 1, and 0 days after ($n = 1$, 3, 5, and 7 nests, respectively) and 1 and 3 days before ($n = 3$ and 1 nests, respectively) clutch completion. To determine how hormone levels changed with regard to the onset of incubation and whether variation in E and P might be explained by its timing, we compared E and P levels across 6 days bracketing the onset of incubation. When nests of all females were analyzed together, E remained relatively unchanged from 3 to 1 days before onset of incubation but dropped approximately threefold on the day incubation began (Fig. 7, left). E levels remained low thereafter. P showed a similar change, though not as marked as that for E.

Birds, such as the canary, that lay one egg per day ovulate the ovum approximately 24 h prior to laying it. Onset of incubation often coincides with laying of the penultimate egg and therefore with ovulation of the last egg. It is therefore conceivable that the hormonal changes shown in Fig. 7 (left) are consequences of a transition from an ovulatory to postovulatory state rather than a transition to incubation behavior. The considerable variation in the onset of incubation with respect to laying of the last egg allowed us to investigate the relationship between hormones and incubation behavior when the relationship between incubation onset and ovulation of the last ovum is uncoupled. To this end, we looked at the changes in E and P relative to the onset of incubation in three groups of females. Females which began incubation on the day before they had laid their last egg (on the day the last egg had been ovulated) or earlier were classified as early incubators. Those which began incubation on the day the last egg had been laid (1 day after the last egg had been ovulated) were middle incubators. Females which began incubation at least 1 day after the last egg had been laid (2 days after the last egg had been ovulated) were late incubators. For each of these three

incubation scenarios, the temporal relationship between E levels and onset of incubation persisted (Fig. 7). That is, E levels dropped sharply at the onset of incubation, irrespective of the termination of ovulation. For middle and late incubators, the relationship between P levels and onset of incubation was also maintained (Fig. 7). However, in early incubators, the decline in P with respect to onset of incubation was more gradual. P declined only partially on the day incubation began and dropped to low levels 1 day after onset of incubation. Error variation in P was relatively large for early incubators, making interpretation of this relationship difficult.

To further address the relationship between hormone levels, onset of incubation, and clutch completion and to potentially reveal hormonal correlates of clutch size regulation, we compared E and P levels across days bracketing laying of the last egg. After analyzing all females together, regardless of the temporal relationship between onset of incubation and clutch completion, we again classified females into the three groups described above. There were statistically significant changes in E and P with respect to clutch completion, but the decline in levels of both hormones did not always occur on the day the last egg was laid (Fig. 8). Specifically, E levels declined the day before the last egg was laid in early incubators, declined the day the last egg was laid in middle incubators, and gradually declined over 3 days in late incubators. P levels declined the day the last egg was laid in early and middle incubators but remained high in late incubators (though the latter relationship was not statistically significant).

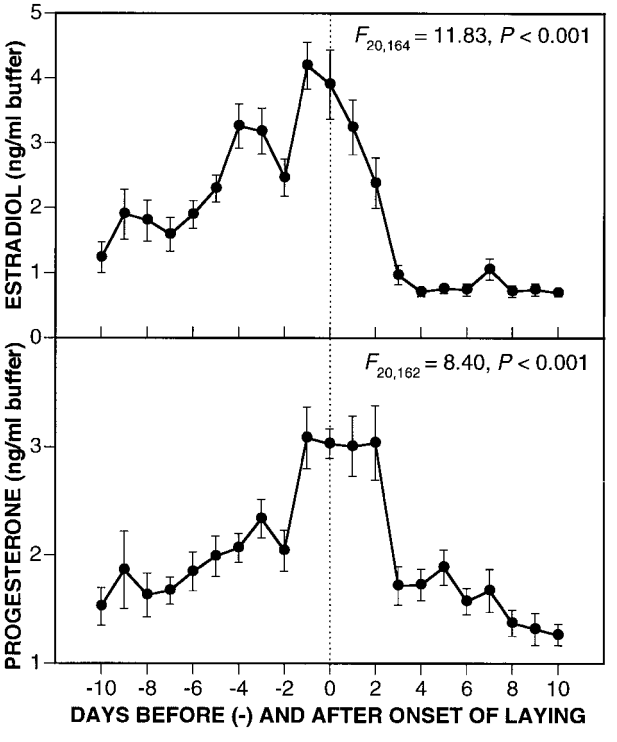

FIG. 5. Immunoreactive estradiol and progesterone in fecal samples collected daily from female canaries before, during, and after laying. Day 0 is when the first egg was laid.

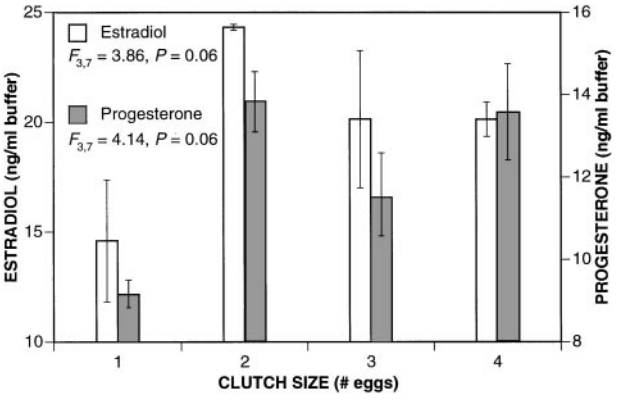

FIG. 6. Immunoreactive estradiol and progesterone in fecal samples from female canaries laying variable clutch sizes. Ordinates are the sums of steroid concentrations from samples collected once at 06:30 each day from 4 days before through 2 days after onset of laying for estradiol and from 1 day before through 2 days after onset of laying for progesterone.

According to linear contrasts, hormone levels before onset of incubation (days -3, -2, and -1) were significantly higher than levels after onset of incubation (days 0, 1, and 2) in all comparisons shown in Fig. 7 ($P < 0.001$). Hormone levels before clutch completion (days -3, -2, and -1) were higher than levels after clutch completion (days 0, 1, and 2) in all comparisons shown in Fig. 8 ($P < 0.001$), with the exception of the comparison of *P* levels in late incubators ($P > 0.20$).

DISCUSSION

We do not know whether changes in fecal E and P reflect differential ovarian production, changes in the metabolism and excretion of these hormones, or a

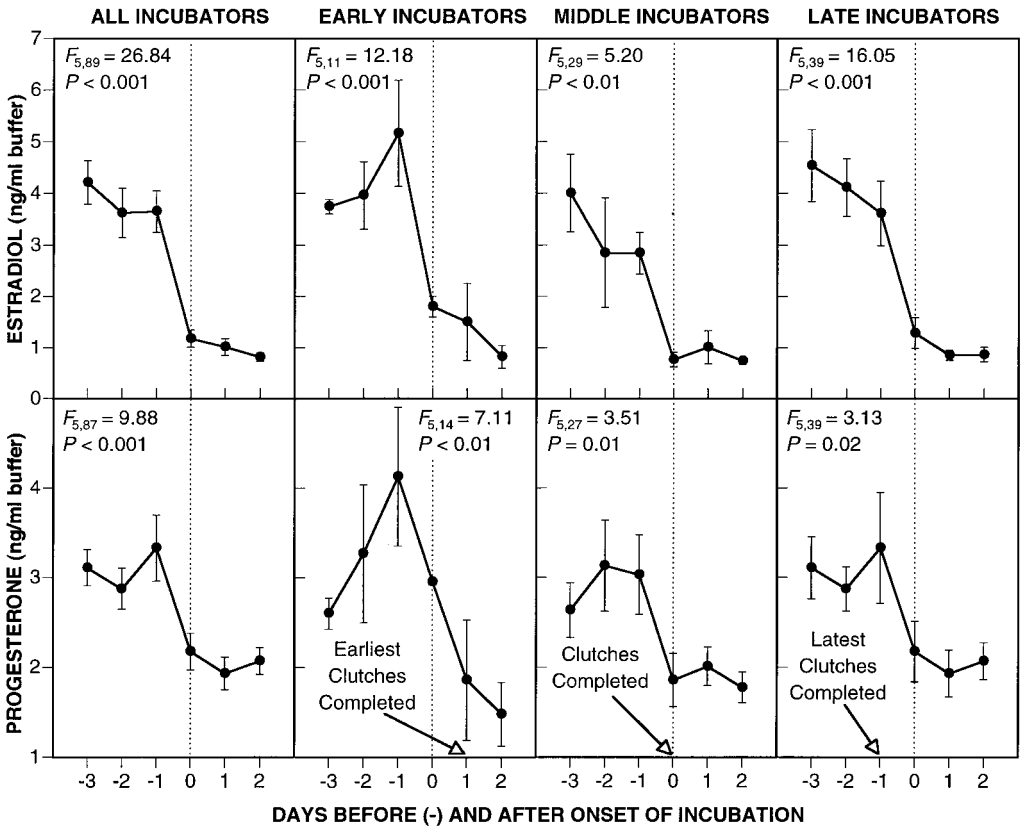

FIG. 7. Immunoreactive estradiol and progesterone in fecal samples from female canaries before and after onset of incubation for all nests and for those nests in which females began incubating before ovulation of the last egg (early incubators), 1 day after ovulation of the last egg (middle incubators), or >1 day after ovulation of the last egg (late incubators). Day 0 is when incubation began.

combination of both. Evidence, however, exists for the former. In male and female Japanese quail (*Coturnix coturnix japonica*) changes in plasma testosterone and P strongly correlate with changes in fecal immunoreactive testosterone and the P metabolite pregnanediol-3α-glucuronide, respectively (Bishop and Hall, 1991). In that study, the fecal concentration of the estradiol metabolite estradiol-3-glucuronide was higher under long than under short photoperiods and was higher in females than in males regardless of photoperiod. This provides physiological evidence that fecal hormone concentrations reflect circulating levels. In breeding canaries, fecal E concentrations were also much higher in females than in males, with no overlap of concentrations between sexes (H. Schwabl, unpublished data). In the present study, the first samples collected in the photophase may have been concentrated because females probably did not excrete during the scotophase. However, fresh fecal samples were usually present before cage papers were changed, indicating that the first collected samples were not the first produced each day. Further, incubating females excreted much less frequently than nonincubating females. Were hormones concentrated during periods of nonexcretion, then a rise in levels at the onset of incubation might be expected. Indeed, the opposite was true. We therefore assume that changes in fecal concentrations reflect changes in ovarian output and plasma levels of these hormones.

A rise in both fecal E and P in female canaries accompanied ovulation of the first egg, whereas correlates of the decline in their levels seemed to differ somewhat between these hormones. For E, the decline accompanied the onset of incubation. For P, the decline seemed more closely associated with time since ovulation of the first egg than with other variables. That is, the abrupt fall in P occurred 4 days after ovulation

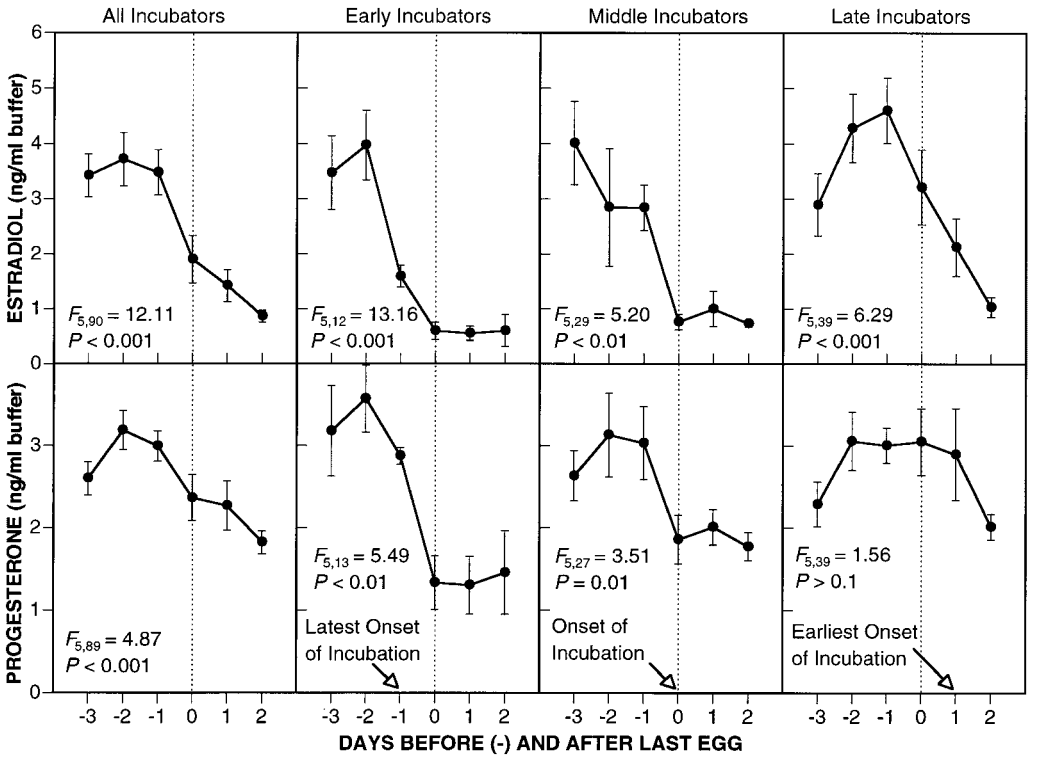

FIG. 8. Immunoreactive estradiol and progesterone in fecal samples from female canaries before and after clutch completion for all nests and for those nests in which females began incubating before ovulation of the last egg (early incubators), 1 day after ovulation of the last egg (middle incubators), or >1 day after ovulation of the last egg (late incubators). Day 0 is when the last egg was laid.

began, regardless of clutch size or nest number. The changes in E levels probably reflect changes early in the photophase only. Results in Fig. 4 suggest that an analysis of E with respect to laying using samples collected later in the day would not reveal any changes in E at all. In other words, E levels 2 or more hours into the photophase were already quite low, regardless of the female's reproductive state (at least within the first 4 d after onset of laying). This may be important for field endocrinologists, who often must rely on samples collected throughout the day.

The sharp rise in E at onset of ovulation and fall at onset of incubation was interesting in light of E's role in egg formation, development of the oviduct, and reproductive behavior. In domestic fowl, stimulation of follicular thecal cells by luteinizing hormone causes production of E which then induces hepatic synthesis and mobilization of major yolk components, such as vitellogenin (Etches, 1996). Exogenous E enhances oviductal growth in fowl, immature Japanese quail (Johnson, 1986; Etches, 1996), and canaries (Follett *et al.*, 1973). E stimulates food intake and deposition of calcium within the medullary portion of long bones (Johnson, 1986), facilitating shell production. Additionally, E may stimulate copulation–solicitation displays in females of some species, including the canary (Kreutzer and Vallet, 1991; Searcy, 1992), though the necessity of E for sexual receptivity remains equivocal (Searcy, 1992; Nagle *et al.*, 1993; Leboucher *et al.*, 1994).

Production of E is highest in small, early-stage follicles and lowest in large, late-stage, yolky follicles (Johnson, 1986; Etches, 1996). One might therefore assume that total ovarian output of E would peak when the number or total E production of small follicles is at a maximum, a time possibly coinciding with onset of ovulation (Fig. 5). Several studies indicate that levels of circulating E are low when females are incubating (see Silver and Cooper, 1983). Our study now reveals the precision with which incubation and the decrease in E levels are coordinated. The day on

which incubation began was marked by an approximately threefold drop in E concentration from the previous day. E remained high after the last egg had been ovulated if incubation had not yet begun and was low during ovulation if incubation had already begun. Together, these findings suggest that until incubation begins a continuous supply of small, E-producing follicles may be present. Though our data are not directly supportive, it is possible that onset of incubation, triggered by a rise in prolactin (see Introduction), may serve to limit the number or steroid output of small follicles. In turn, development of large follicles may arrest under the duress of low circulating yolk components, possibly preventing their eventual ovulation.

The rise in P leading up to ovulation and laying is consistent with the putative relationship between P and ovulation. In fowl, a rise in P, which is produced by large, yolky follicles, stimulates a surge in luteinizing hormone, which triggers ovulation (Sharp, 1980). Although P declined with onset of incubation, inspection of Figs. 5 and 7 suggests that a more substantial fall occurred 3 days after onset of laying among females laying different clutch sizes. Further, P levels were not related to clutch size and were high in some females well after clutch completion, a time when ovulation had presumably ended. It therefore seems plausible that the ovary is set to grow the four follicles necessary for the canary's modal clutch size of four eggs, as supported by the 4-day plateau in P beginning with ovulation of the first ovum. Control by the hypothalamus or pituitary, possibly via reduced production of E from small follicles, may prevent ovulation of some large follicles, thus influencing clutch size. However, this does not explain why some females in our study completed clutches before incubation began. Further studies on follicular number, size, and steroid production in relation to incubation onset and studies in which incubation onset is experimentally induced or prevented are needed to determine the relationship between onset of incubation, steroid output, and clutch size.

Mead and Morton (1985) proposed that onset of incubation and clutch size determination are hormonally coupled. Meijer *et al.* (1990) went on to suggest that an increasing tendency to incubate may serve to regulate the seasonal decline in clutch size observed in many species. Our results on the change in ovarian steroid hormones in relation to onset of incubation raises the possibility of an additional means by which females may optimize reproduction. In canaries and other birds, androgens are transferred into egg yolks (Schwabl, 1993). These yolk androgens modify offspring development and aggression (Schwabl, 1993, 1997) and may reflect diverse reproductive strategies (Schwabl *et al.*, 1997). Should high levels of circulating steroids be maintained throughout laying by a late onset of incubation, later laid eggs might receive greater doses of them than were incubation to begin early. In late-incubating females, high hormone levels in egg yolks might then confer advantages to later-laid eggs already benefiting from a more synchronous onset of incubation. In a pattern similar to what we show here for E, fecal testosterone in laying females remains high until incubation begins and positively correlates with yolk levels (Schwabl, 1996). Whether variable timing for the onset of incubation causes corresponding changes in concentrations of yolk steroids has yet to be determined.

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