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Removing the confound of time in investigating the regulation of serial behaviours: testosterone, prolactin and the transition from sexual to parental activity in male American kestrels

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Understanding the relationships among variables can be difficult if the variables covary with time. Using a simple, commonly used statistical procedure to control for the passage of time, we demonstrate that some hormonal changes traditionally assumed to be causally related to the transition from sexual to parental behaviour can be explained by seasonal changes in the hormones and the fact that parental behaviour follows sexual behaviour in the course of breeding. We quantified immunoreactive prolactin (ir-prolactin) and testosterone concentrations in laboratory-housed and free-living male American kestrels, *Falco sparverius*, over the course of the breeding season and during sexual and parental phases of reproduction. We found that ir-prolactin increased while testosterone decreased with the transition from sexual to parental behaviour. ir-Prolactin increased with date (i.e. seasonally), whereas testosterone peaked when the frequency of clutch initiation was greatest and then decreased with date thereafter. Controlling for the effects of date eliminated the change in ir-prolactin associated with the transition from sexual to parental behaviour, revealing that prolactin variation associated with this behavioural transition in male kestrels is due, in large part, to seasonal changes in hormone concentrations and the serial nature of these behavioural states. Controlling for the effects of date further revealed that the testosterone difference between sexual and parental males decreases seasonally. It is possible that other phenomena traditionally attributed to environmental, behavioural, social, or other variables also may be explained by the association between such variables and the passage of time.

Throughout all levels of biological organization, understanding the relationships among variables can be difficult if the variables covary with time or other variables. For example, the transition from sexual to parental behaviour in vertebrates is often associated with a decline in plasma testosterone and concomitant elevation in plasma prolactin concentrations. Concentrations of these hormones also change with time, in particular over the course of a breeding season. This raises the possibility that, in some species, the change in these hormone concentrations may be a result, not of the behaviours themselves, but rather of the serial nature of these behaviours and of seasonally programmed hormone cycles.

Experimental manipulations help to elucidate the cause-and-effect relationships among variables and can control for time as a confound in some cases. However, some variables (in particular, behaviour) are difficult to manipulate or control, and the question often remains as to whether effects of the experimental manipulation are direct or indirectly mediated through secondary mechanisms. The purpose of this study is (1) to illustrate the importance of considering the potential confound of time when investigating the regulation of serial behaviours, which themselves cannot be directly manipulated, (2) to demonstrate that a simple, commonly used (in some fields) statistical procedure sometimes can remedy this confound, and (3) to demonstrate that hormonal dynamics previously assumed to be causally related to the

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transition from sexual to parental behaviour are, in some cases, a product of the time of the breeding season during which these behaviours are most likely to occur.

In male temperate-zone birds, the onset of the breeding season is generally associated with elevated plasma concentrations of the sex-steroid hormone testosterone (Wingfield & Farner 1980; Wingfield 1984). An increasing photophase for long-day breeders precipitates an initial rise in circulating testosterone concentrations, which rise further during aggressive interactions with other males and courtship and sexual interactions with females (Schlinger et al. 2001). In socially monogamous species, testosterone concentrations are typically low after egg laying and during the ensuing periods of parental care (Silver 1978; Wingfield et al. 1990; Seiler et al. 1992), a time when otherwise elevated testosterone may interfere with parental behaviour such as incubation and feeding of the young (see Schoech et al. 1998; Ketterson & Nolan 1999; McDonald et al. 2001). In some species in which transient elevations in testosterone may occur during paternal care (Wingfield et al. 1990), the sensitivity of individuals to elevated testosterone at this time may vary with the benefits of biparental care relative to sexual behaviour during parental phases of reproduction (Hunt et al. 1999; Lynn et al. 2002; Van Duyse et al. 2002).

Elevated concentrations of the pituitary hormone prolactin are associated with periods of parental care (Dixson & George 1982; Brown 1993; Buntin 1996) and, in birds, may enhance or maintain such parental behaviours as incubation (Goldsmith 1983; Lea et al. 1986) and feeding of the young (Lea 1987; Buntin et al. 1991). In species in which males partake in these parental behaviours, plasma prolactin concentrations in males are usually relatively low during territory acquisition and times when sexual activities such as courtship and copulation predominate (Oring et al. 1986; Williams & Sharp 1993; Vleck et al. 1999). Prolactin concentrations then tend to rise prior to and during the subsequent incubation stage (Hector & Goldsmith 1985; Goldsmith 1991; Sharp et al. 1997; Buntin et al. 1998). In short, the transition in males from sexual behaviour such as courtship to parental behaviour such as incubation is typically associated with a decline in testosterone concentrations and concomitant elevation in prolactin concentrations (e.g. Seiler et al. 1992; Vleck et al. 1999; Lormée et al. 2000; Khan et al. 2001).

Could this association between hormone concentrations and reproductive behaviour be a result of photo-induced, seasonal changes in hormones and the passage of time between reproductive behaviours? Like testosterone, prolactin also shows a photo-induced rise (Dawson & Goldsmith 1983; Follett 1984; Nicholls et al. 1988). Because this rise may occur in males that are somatically immature (Sreekumar & Sharp 1998a), laboratory-housed without mates (Silverin & Goldsmith 1997; Dawson & Sharp 1998; Sreekumar & Sharp 1998b), or engaged in only nonparental behaviour (Dawson & Goldsmith 1985; Maney et al. 1999), elevations in prolactin over the breeding season may, in part, be independent of parental behaviour. Many studies that have reported hormonal changes associated with the transition from sexual to parental behaviour have not excluded the possibility that these changes may be a result of seasonal changes in these hormone concentrations that coincide with a population-wide mean time during which these behaviours occur. That is, hormonal changes associated with the transition from sexual to parental behaviour may be an artefact of seasonal changes in these hormones (i.e. the passage of time) because these hormone concentrations change seasonally and because parental behaviour necessarily follows sexual behaviour in the course of breeding.

Dawson & Goldsmith (1985) addressed this problem by removing eggs from the nests of European starlings, *Sturnus vulgaris*, to induce renesting, while allowing others to progress normally through the reproductive cycle. This desynchronized the population, enabling a comparison of hormone concentrations between sexual and parental males at the same time of the season. Their findings suggest that both date and reproductive behaviour contribute to patterns of prolactin and testosterone secretion. However, because the timing of laying behaviour was not (and cannot be) directly manipulated, it is not possible to know the extent to which hormonal concentrations were influenced by egg removal, by renesting itself, or by the behaviour of interest. An alternative approach to examining hormonal associations with reproductive behaviour in populations that are naturally asynchronous is to compare hormone profiles between sexual and parental individuals at a single point in time or to statistically remove seasonal effects on these hormones from changes associated with the transition from sexual to parental behaviour. To that end, we investigated in laboratory-housed and free-living male American kestrels, *Falco sparverius*, variation in plasma concentrations of immunoreactive prolactin (ir-prolactin) and testosterone associated with both date (i.e. seasonal change or change with the passage of time) and the transition from sexual to parental behaviour. Taking advantage of the highly asynchronous breeding phenology of the American kestrel, we controlled for seasonal variation in ir-prolactin and testosterone to determine whether changes in these hormones associated with the transition from sexual to parental behaviour occurred independently of the passage of time.

The American kestrel is a socially monogamous falcon. Indicative of their highly asynchronous breeding phenology, pairs in our free-living study population initiate laying from late March through to late June (Sockman & Schwabl 2001). Prior to and during laying, males provision females with food, a courtship activity that may secure the pair bond and help stimulate laying in females (Balgooyen 1976). Courtship feeding is accompanied by vocalizations directed towards the female and prodigious mounting behaviour well before the onset of laying, which may also help to secure the pair bond. Females usually lay every 2 days until the clutch is complete at 4–6 eggs. Incubation and nestling periods, each approximately 30 days, follow, after which nestlings fledge (Balgooyen 1976; Sockman & Schwabl 1998). Males and females both engage in parental care including incubation and feeding of the young, and both sexes may, in fact, engage in parental behaviour (incubation) prior to the complete cessation of sexual behaviour during laying (Balgooyen

1976; Bortolotti & Wiebe 1993). Thus, before laying, sexual behaviour predominates, and after laying parental behaviour predominates. During laying itself, both behaviours may occur. Laying may therefore be thought of as a transitional phase during which sexual behaviour is waning and parental behaviour is developing simultaneously. If prolactin and testosterone are respectively involved in mediating parental and sexual behaviour in the American kestrel, plasma concentrations of both hormones should be relatively elevated during laying.

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, U.S.A.) for the humane treatment of our subjects. We previously outlined our general laboratory and field procedures, including information on the age and source of animals and on housing conditions (Sockman & Schwabl 2000; Sockman et al. 2000). Below we describe procedures specific to this study.

Laboratory Study

We randomly formed 18 male–female pairs of American kestrels (in individual pens within one room in which pairs could hear but not see each other), held them on an 8:16 h light:dark cycle, provided them with water, and fed them three to four 1-day-old cockerel chicks per day (ad libitum). On 24 January 1998 we changed the photoperiod to LD 10:14 h, on 31 January to LD 12:12 h, and on 7 February to LD 14:10 h (similar to late April at the latitude of the field study). Beginning with the change to LD 12:12 h, we randomly assigned nine pairs to a moderately reduced food availability of two cockerel chicks/day. We maintained the other nine on ad libitum food availability. We returned food-reduced pairs to ad libitum food availability on 3 April 1998, a point 9 weeks after transfer to LD 12:12 h and after which all pairs had completed clutches. We conducted this food-reduction treatment as part of another study on the role of food availability in the reproductive behaviour and endocrinology of these males' mates (Sockman et al. 2000, 2001; Sockman & Schwabl 2001). Here, we do not discuss results pertaining to the effects of food treatment, because these effects (or lack thereof) were not of primary interest in the present study. However, we included food treatment as a factor in the laboratory statistical analyses because food availability has been shown to influence prolactin concentrations (Millam & El Halawani 1986; Sockman et al. 2001).

We collected ca. 400-μl blood samples from alar veins of males every 14 days, beginning immediately before the first increase in the photophase and lasting through the 12 weeks during which we maintained them on an LD 14:10 h cycle. The time at which their mates initiated laying varied among pairs, and, depending on the pair, this biweekly collection of blood samples often coincided with prelaying, laying, or incubation behaviour (see definitions below). This asynchrony in behaviour enabled us to separate the effects of date from those of behaviour on hormone concentrations (see below). We stored blood on ice for a few hours before centrifugation at 9000 revolutions/min for 9 min to separate plasma, which we stored at –20°C.

Field Study

During the 1997 and 1998 field seasons, we investigated variation in ir-prolactin and testosterone concentrations relative to date and reproductive behaviour in free-living, paired males using nestboxes we placed along roadsides within 35 km of the Washington State University campus (Pullman, Washington, U.S.A.; 46°44′N, 117°10′W). Under licence from the U.S. Fish and Wildlife Service (permit number 22 769) and from the Washington Department of Fish and Wildlife (permit number 99-058), we captured males in nestboxes or with a bal-chatri trap (Berger & Mueller 1959), banded them, and collected blood samples of 400–600 μl, which we processed as described above.

Hormone Measurements

ir-Prolactin was measured in duplicates of 25 μl plasma diluted to a 100 μl volume in one radioimmunoassay, using antisera against recombinant-derived starling prolactin, and following the protocol of Bentley et al. (1997). The intra-assay 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 Figure 1 in Sockman et al. 2000 for statistics and serial dilution curves).

To measure plasma testosterone, we randomized the order of our samples between two radioimmunoassays (interassay coefficient of variation = 7.8%, intra-assay coefficients of variation = 3.2 and 3.9%), each with a sensitivity of 1.95 pg/tube. We added tritiated testosterone (2000 counts/min) to each plasma sample (100 μl) for estimation of recoveries (mean = 60%, range 51–73%) and allowed them to equilibrate overnight at 4°C. We extracted steroids with 2×4 ml of petroleum ether and diethyl ether (3:7 by volume) using Extrelut (EM Science, Gibbstown, New Jersey, U.S.A.) minicolumns and dried extracts under a stream of nitrogen. We separated and partially purified testosterone on diatomaceous earth chromatographic columns as described by Schwabl (1992). We dried testosterone fractions under nitrogen and redissolved them in phosphate-buffered saline (pH 7.1). The remainder followed a previously published radioimmunoassay protocol (e.g. Schwabl 1995).

Definitions of Reproductive Stages and Analytical Approach

We obtained plasma samples in the laboratory study for three reproductive stages, prelaying, laying and incubation, and, in the field study, for two reproductive stages, laying and incubation. We obtained a plasma sample from

one male in the field during prelaying, which we used only in the analyses involving seasonal but not behavioural changes in hormones. We had no samples from males with nestlings and therefore limit our analyses of parental behaviour to the incubation stage. Several studies on males of other species, which have shown that prolactin rises and testosterone declines with the transition from the laying to the incubation stage, have failed to show that prolactin rises further or testosterone declines further with the transition from the incubation to the nestling stage (e.g. Seiler et al. 1992; Lormée et al. 2000; Khan et al. 2001). Therefore, it is likely that our comparisons between sexual and incubating (but not nestling-feeding) males captured the most significant behavioural transition in terms of hormonal change.

We defined prelaying as the 10-day period preceding laying of the first egg of a clutch and laying as the period during which the male's mate was laying a clutch of eggs. We defined incubation as the period from the day on which the last egg of a clutch was laid through to 15 days after clutch completion (approximately midway through the incubation stage). By the time the last egg has been laid, incubation behaviour (in males and females) is near full expression (Sockman et al. 2000) and sexual (i.e. copulation) behaviour is negligible. Although our laboratory males rarely incubated during laying, free-living males often do incubate during this time, and sexual behaviour in both environments rapidly declines. We collected few plasma samples from renesting males, so we limited all analyses to first nests.

We first present results from analyses in which we investigated the role of date and, in separate analyses, the role of reproductive behaviour on prolactin and testosterone. This allowed us first to show that these hormones vary seasonally, and second, to illustrate the conclusions one could make on the relationship between hormone concentrations and reproductive behaviour in the absence of considering the role of date. We subsequently present results from analyses on the relation between reproductive behaviour and hormone concentrations while controlling for the effects of date. Below we provide details for each of these analytical approaches. In figures, we plot adjusted (least squares) means from the models described below to illustrate changes in hormone concentrations with and without date as a factor in the statistical model.

SEPARATE EFFECTS OF DATE AND REPRODUCTIVE BEHAVIOUR

Analysis

To investigate the effects of date on ir-prolactin and testosterone concentrations in the laboratory, we conducted repeated measures analyses of variance with day as a within-subjects factor (and food treatment as a between-subjects factor; see above). We investigated change in ir-prolactin and testosterone concentrations with respect to reproductive behaviour in the same manner, except that reproductive stage was the within-subjects factor. For the field study, we analysed variation in ir-prolactin and testosterone concentrations with respect to date using linear regressions and with respect to reproductive stage using analyses of variance (ANOVA). Because we had fewer plasma samples collected in the field during incubation than during laying, we selected from each of the three males from which we had more than one sample that sample collected during incubation (to help balance the sample sizes), thus analysing only one sample per male.

Results

Laboratory study

The mates of 16 (8 control and 8 food-restricted) of the 18 males laid complete clutches. To ensure that our analyses included only reproductively viable pairs, we excluded the two males whose mates never laid. On some occasions, we did not obtain adequate blood samples, causing our sample sizes to vary with day of the study.

Both ir-prolactin (ANOVA: $F_{7,88} = 86.56$, $P < 0.0001$) and testosterone (ANOVA: $F_{7,88} = 8.55$, $P < 0.0001$) concentrations varied significantly with day (Fig. 1a, b). ir-Prolactin and testosterone were low at the onset of the study, when males had been exposed to an LD 8:16 h photoperiod. By day 28 (14 days after onset of LD 14:10 h), a point 13 days prior to initiation of the first clutch, both ir-prolactin and testosterone had increased significantly. ir-Prolactin continued to rise until day 70, at which point it reached a plateau and remained high until day 98. Testosterone rose to a maximum at day 42, a point corresponding to the peak period for clutch initiation (Fig. 1c). Thereafter, testosterone declined, as did the frequency of clutch initiation.

Among the plasma samples collected every 14 days, nine were collected during prelaying, 10 during laying and 12 during incubation. Because the incubation stage (as we defined it) lasted more than 14 days, we had two incubation samples for a few males. In such cases we used only the first collected sample for analyses. The effects of reproductive stage on both ir-prolactin (ANOVA: $F_{2,11} = 7.40$, $P = 0.009$) and testosterone concentrations (ANOVA: $F_{2,11} = 23.09$, $P = 0.0001$) were statistically significant. ir-Prolactin increased from prelaying to laying and from prelaying to incubation (Fig. 2a, circles). Differences between laying and incubation concentrations were not statistically significant. Testosterone concentrations declined from prelaying and laying to incubation (Fig. 2b, circles). Differences between prelaying and laying concentrations were not statistically significant.

Field study

Over the course of the breeding season, ir-prolactin concentrations increased (linear regression: $F_{1,24} = 6.35$, $P = 0.019$, $R^2 = 0.21$), whereas testosterone concentrations decreased (linear regression: $F_{1,24} = 4.76$, $P = 0.039$, $R^2 = 0.17$; Fig. 3a, b). Date of clutch initiation was highly asynchronous, spanning a period from early April through to late June (Fig. 3c).

We analysed 14 plasma samples collected during the laying stage and eight collected during the incubation stage. For three males in the above analysis on the effects

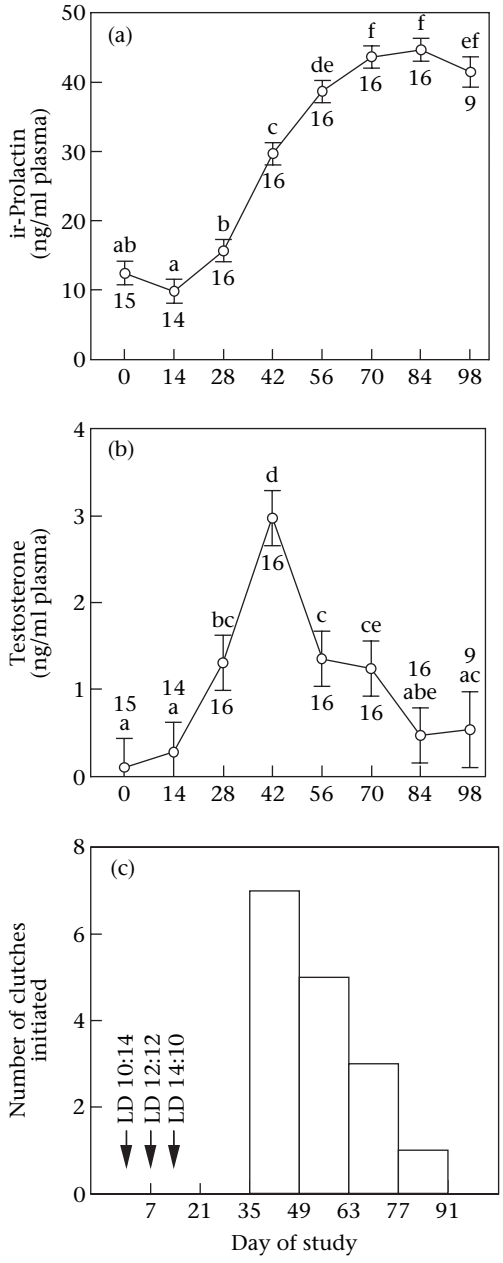

Figure 1. Photo-induced seasonal change in (a) immunoreactive prolactin and (b) testosterone in laboratory male American kestrels, and in (c) frequency of clutch initiation by their mates. Hormone concentrations are adjusted (least squares) means (±SE) from the ANOVAs described in the text. Clutch initiation times (the day on which the first egg of a clutch was laid) were divided into 14-day intervals centred around each of the days for which hormone concentrations are depicted. Mated pairs were held in individual pens on an 8:16 h light:dark cycle until day 0 of the study, at which point the photoperiod was stepped up to LD 14:10 h by 2-h intervals per week as indicated by the arrows in (c). Plasma samples for hormone analyses were collected immediately before pairs experienced the change in photoperiod that occurred on that day. Thus, day 0 hormone concentrations reflect those of birds on an LD 8:16 h photoperiod. Points with a letter in common were statistically indistinguishable at an α of 0.05 based on post hoc linear contrasts. Numbers above or below each point indicate the sample size of males.

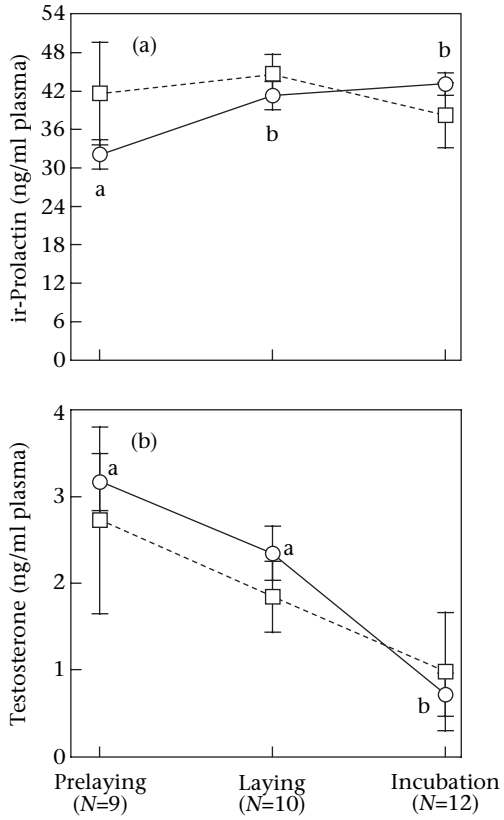

Figure 2. Change in (a) immunoreactive prolactin and (b) testosterone with respect to reproductive stage in laboratory male American kestrels. Hormone concentrations are adjusted (least squares) means (±SE) from ANOVAs with week of experiment as a factor (□) and without week of experiment as a factor (○) in the model, as described in the text. Points with a letter in common were statistically indistinguishable at an α of 0.05 based on post hoc linear contrasts conducted where the main effect of reproductive stage was statistically significant. Sample size of males is given for each reproductive stage.

of date, we did not know the reproductive stage at the time we collected blood samples, although we did know that samples were collected during either laying or incubation. (As mentioned above, one sample was collected during prelaying and was therefore not used below.) We did not include these three males in the following analyses. ir-Prolactin concentrations did not change significantly with reproductive stage (ANOVA: $F_{1,20} = 3.08$, $P = 0.095$; Fig. 4a, solid bars). Testosterone was high during the laying stage and then dropped significantly (ANOVA: $F_{1,20} = 6.33$, $P = 0.020$) during the incubation stage (Fig. 4b, solid bars).

EFFECTS OF REPRODUCTIVE BEHAVIOUR WHEN CONTROLLING FOR EFFECTS OF DATE

Analysis

It is possible that the change in hormone concentrations associated with the transition from sexual to parental behaviour shown in Fig. 2 (circles) and Fig. 4b

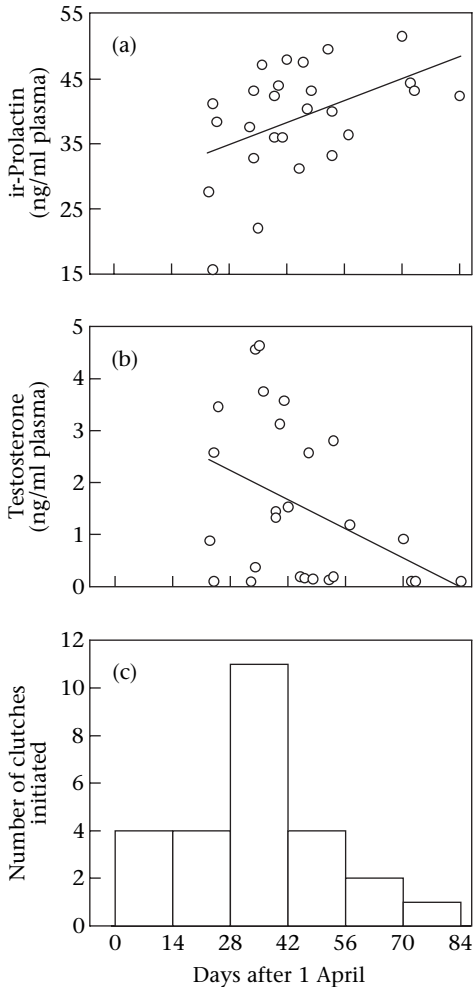

Figure 3. Seasonal change in (a) immunoreactive prolactin and (b) testosterone in free-living male American kestrels and in (c) frequency of clutch initiation by their mates.

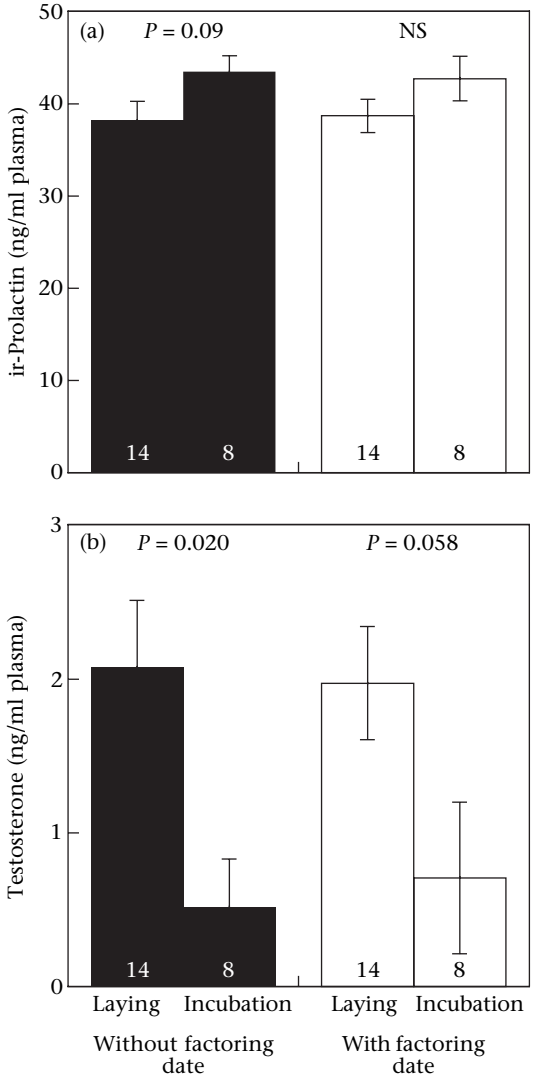

Figure 4. Change in (a) immunoreactive prolactin and (b) testosterone with respect to reproductive stage in free-living male American kestrels. Hormone concentrations are adjusted (least squares) means (±SE) from ANOVAs with (□) and without (■) date as a factor in the model. Numbers at the base of bars indicate the sample size of males for each reproductive stage.

(solid bars) is a result of changes associated with date shown in Figs 1 and 3 (see Introduction). This possibility becomes all the more apparent when considering the fact that most clutches in the laboratory study were initiated at roughly the time in the breeding phase when ir-prolactin concentrations had already increased significantly and when testosterone concentrations were maximal as shown in Fig. 1. Asynchrony in reproductive phenology enabled us to control for the passage of time when assessing hormonal changes associated with reproductive stage. We did so using two different analytical approaches. In the first, we selected a single point in time to compare hormone concentrations between sexual and parental males. Thus, in this initial analysis, we selected day 56 of the laboratory study, because it was the only time point (in either study) in which sample sizes of sexual ($N = 5$ prelaying) and of parental ($N = 5$ incubation) males were sufficient for a meaningful comparison. The disadvantage of this approach is that it forced us to ignore potentially important data from other points in time. To that end, we took the additional approach as follows. To the analysis of variance models addressing reproductive stage described above, we added the covariate day to control for the possible role that the passage of time might have on behavioural-state changes in hormone concentrations. We used type III sums of squares, which remove the effects of all other factors in the model before testing the factor in question. Therefore, in an analysis of covariance with day as a covariate, the F statistic for reproductive stage reflects variation in hormone concentrations explained by reproductive stage after the effects of day have been removed.

Some comparisons among laboratory and field results led to ambiguous interpretations (see below), possibly due to low statistical power. Therefore, in an additional analysis, we combined laying and incubation data from the laboratory and field studies in two multifactor analyses of covariance, one for ir-prolactin and one for testosterone. The factors in each model included study (to remove potential differences between the laboratory and

field studies), reproductive stage (laying versus incubation), day (of blood collection), and all interactions. We standardized the term day of blood collection between the two studies by subtracting the day of blood collection from the day on which the first egg of the study was laid.

Results

Laboratory study

At day 56 of the laboratory study, prelaying ir-prolactin concentrations did not differ from incubation concentrations (ANOVA: $F_{1,6} = 1.09$, NS; Fig. 5a), however, prelaying testosterone concentrations were significantly higher than incubation concentrations (ANOVA: $F_{1,6} = 18.9$, $P = 0.005$; Fig. 5b). When we used data from throughout the study and included day as a covariate in the model, the effect of reproductive stage on ir-prolactin concentrations was not statistically significant (ANCOVA: $F_{2,8} = 0.77$, NS). That is, ir-prolactin did not change from prelaying to laying to incubation behaviour (Fig. 2a, squares). The effects of reproductive stage on testosterone concentrations were also statistically nonsignificant (ANCOVA: $F_{2,8} = 2.12$, $P = 0.18$), although, unlike ir-prolactin, the pattern of change was similar to that when not controlling for date (Fig. 2b, squares).

Field study

With date included as a covariate in the model, the effect of reproductive stage on ir-prolactin concentrations was not statistically significant (ANCOVA: $F_{1,19} = 1.70$, NS). ir-Prolactin did not change from laying to incubation behaviour (Fig. 4a, open bars). With date included as a covariate, testosterone concentrations during laying were higher than those during incubation (ANCOVA: $F_{1,19} = 4.07$, $P = 0.058$), with a pattern of change similar to that when not controlling for date (Fig. 4b, open bars).

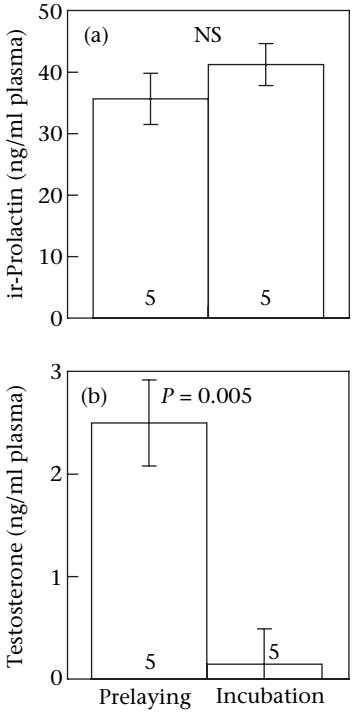

Figure 5. Change in (a) immunoreactive prolactin and (b) testosterone with respect to reproductive stage in male American kestrels at day 56 of the laboratory study. Hormone concentrations are adjusted (least squares) means (±SE) from the ANOVAs described in the text. Numbers at the base of bars indicate the sample size of males for each reproductive stage.

Combined laboratory and field studies

When we combined laying and incubation data from the laboratory and field studies, neither the term study nor any interactions with study affected either ir-prolactin or testosterone concentrations (Table 1). This indicates that any potential differences between the laboratory and field studies could not explain variation in hormones (study main effect), variation in the way in which the hormones changed with respect to day (study∗day interaction) or reproductive stage (study∗reproductive stage interaction), or variation in the way in which the difference associated with reproductive stage changed with respect to day (study∗day∗reproductive stage interaction).

Day of blood collection explained variation in ir-prolactin, but reproductive stage and the day∗reproductive stage interaction did not (Table 1, Fig. 6a). Day of blood collection negatively correlated with testosterone concentrations, and testosterone concentrations were significantly higher during laying than during incubation (Table 1, Fig. 6b). However, this effect of behaviour on testosterone varied seasonally, as indicated by the significant interaction between day of blood collection and reproductive behaviour (Table 1). Further post hoc examination of this interaction revealed that testosterone declined with day of blood collection during the laying phase (ANCOVA: $F_{1,20} = 7.73$, $P = 0.012$) but, regardless of day of blood collection, remained low during the incubation phase (ANCOVA: $F_{1,16} = 0.03$, NS) (Fig. 6b). Removal from analyses of the possible outlier testosterone value of 11.9 ng/ml (see Fig. 6b) had no effect on whether or not a term in the model was statistically significant ($P < 0.05$).

Table 1. ANCOVA (type III sums of squares) statistics indicating the effects of time (day) and behaviour (stage) on immunoreactive prolactin and testosterone in male American kestrels breeding in the laboratory and field (study)

Source of variation	*df*	ir-Prolactin MS	ir-Prolactin *F*	ir-Prolactin *P*	Testosterone MS	Testosterone *F*	Testosterone *P*
Day	1	265.71	7.17	0.011	20.77	6.83	0.013
Stage	1	47.49	1.28	0.265	27.93	9.18	0.004
Study	1	19.89	0.54	0.469	0.29	0.10	0.758
Day∗stage	1	94.73	2.56	0.119	22.23	7.31	0.010
Day∗study	1	17.28	0.47	0.499	5.80	1.91	0.176
Stage∗study	1	15.02	0.41	0.528	0.80	0.26	0.612
Day∗stage∗study	1	134.84	3.64	0.064	6.21	2.04	0.162
Residual	36	37.08			3.04		

Study was included as a factor to control for potential differences between the laboratory and field.

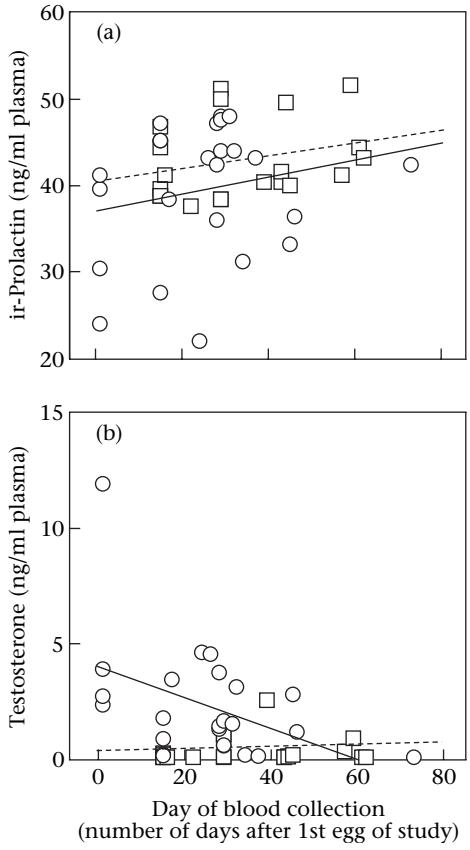

Figure 6. Change in (a) immunoreactive prolactin and (b) testosterone in laboratory and free-living male American kestrels combined. Data are separated for males whose mates were in the laying phase (–○–) and males during the incubation phase (-□-). Day of blood collection was standardized between the two studies by subtracting the day of blood collection from the day on which the first egg of the study was laid.

DISCUSSION

The elevation in prolactin concentrations associated with the transition from sexual to parental behaviour (Fig. 2a, circles) in male American kestrels is not due to the behavioural transition itself, but rather to a seasonal increase in prolactin (Figs 1a, 3a, 6a) and the fact that parental behaviour necessarily follows sexual behaviour (cf. Fig. 2a, circles and squares). Much of the support for this contention rests on data from the laboratory study, due to our lack of prelaying (sexual only) data in the field and the fact that we did not expect large changes in prolactin associated with the transition from laying to incubation (see Introduction).

The passage of time also influences the association between testosterone and the transition from sexual to parental behaviour but in a manner that differs from its influence on the association between prolactin and this behavioural transition. Testosterone was universally low during parental behaviour, regardless of date (Fig. 6b, squares). However, it was high during sexual behaviour early (Figs 5b, 6b, circles) but not late (Fig. 6b, circles) in the season, resulting in a seasonal decline in the difference in testosterone between the sexual and parental phases of reproduction. These findings underscore the importance of controlling for the passage of time in investigating the regulation of serial behaviours.

Our observation that prolactin concentrations increased with date (Figs 1a, 3a, 6a) is consistent with other studies (e.g. Silverin & Goldsmith 1997; Dawson & Sharp 1998). Similarly, our observation that testosterone concentrations increased to a sharp peak around the time when frequency of clutch initiation was greatest (Fig. 1b, c) and then declined thereafter (Figs 1b, c, 3b, c, 6b) is expected for single-brooded species with a high level of paternal care (see Figure 4 in Wingfield et al. 1990). Confirmation of these seasonal changes in both prolactin and testosterone in reproductively active male kestrels served as the basis for our argument that such changes may account for the hormonal dynamics associated with the transition from sexual to parental behaviour, which themselves necessarily occur sequentially.

Indeed, we found that the transition from sexual to parental behaviour was associated with a rise in prolactin (Fig. 2a, circles) and a decline in testosterone (Figs 2b, circles, 4b, 5b, 6b) concentrations, as has also been shown previously in male birds that participate in parental care (see Introduction for citations). Of interest, however, is the laboratory finding that the change in prolactin concentrations between behaviours was largely dependent upon the passage of time and the fact that parental behaviour follows sexual behaviour. We demonstrated this by statistically removing the effects of date, thereby eliminating the increase in prolactin concentrations that otherwise occurs with the transition to parental behaviour (Fig. 2a, squares). It might be argued that, with the addition of the variable date (and the consequent loss of an error degree of freedom), we lacked sufficient statistical power to demonstrate an effect of behaviour on prolactin. However, inspection of Fig. 2a suggests otherwise. When the effects of time were removed (by adding the factor date to the model), not only did prolactin not significantly increase with the behavioural transition, the pattern of change was noticeably altered, suggesting that the passage of time contributed to the significant behaviour effect seen in Fig. 2a (circles).

It is possible that further adjustments in prolactin secretion occur as a result of factors in addition to date (i.e. presence of eggs, young, or a sexually receptive mate) and that the magnitude of these adjustments was below our ability to detect them. In fact, it is generally assumed that in female birds, the presence of eggs or young directly stimulates prolactin secretion (see Goldsmith 1983). Stimuli controlling prolactin secretion in male birds are less well understood; however, as mentioned previously, Dawson & Goldsmith (1985) found evidence in European starlings that both date and reproductive stage modify prolactin secretion in males (and females). In the cooperatively breeding Florida scrub-jay, *Aphelocoma c. coerulescens*, prolactin concentrations are more closely associated with reproductive stage than with date (Schoech et al. 1996). In contrast, elevation of prolactin in male Adélie penguins, *Pygoscelis adeliae*, is relatively independent of tactile input from eggs and chicks and is instead part of a programme of cyclical hormonal changes associated with a long breeding

season (Vleck et al. 2000). Similar findings were reported for albatrosses (Hector & Goldsmith 1985). Thus, it appears that the relative roles of date and reproductive behaviour on prolactin concentrations vary among species and may depend on the species-specific breeding strategy or environment (but see Khan et al. 2001).

With respect to testosterone regulation, results from the laboratory and field studies, when analysed separately, were somewhat ambiguous. At day 56 of the laboratory study, the difference in testosterone concentrations between sexual to parental males was clear (Fig. 5b), indicating that, when there was no passage of time, changes in testosterone were associated with this behavioural transition, at least for that time of the season. Conversely, removing the effects of the passage of time on testosterone concentrations over the course of the entire study in the laboratory eliminated the statistically significant change that otherwise occurred with the transition from sexual to parental behaviour (Fig. 2b) and rendered this change marginally nonsignificant in the field (Fig. 4b, open bars). Yet, unlike the case for prolactin, the patterns of change in the laboratory and field analyses did not change appreciably, raising the possibility that the lack of a behaviour effect with the inclusion of date in the model could be conditional, due to a loss of statistical power, or both. The more statistically powerful analysis combining field and laboratory data revealed that behaviour and date simultaneously influenced testosterone and, in addition, that the effects of behaviour depended on date. In other words, testosterone differed between sexual and parental males early but not late in the season. This is because testosterone declined with date of sexual behaviour but was universally low during parental-only behaviour, regardless of date (Fig. 6b). Thus, in contrast to the effects of the passage of time on prolactin concentrations, the passage of time did not give rise to the change in testosterone associated with the transition from sexual to parental behaviour. Rather, time modulated the decline in testosterone with this behavioural transition.

These findings that the passage of time may influence hormonal changes associated with the transition between sexual and parental behaviour raise questions about the cause-and-effect nature of prolactin, testosterone, and this behavioural transition. The behavioural effects of elevated prolactin and testosterone in male American kestrels are not known. However, as indicated in the Introduction, a number of studies on other bird species suggest that elevated testosterone in males promotes courtship and sexual behaviour directed towards females. Elevated prolactin in laying female kestrels promotes parental behaviour in the form of incubation (Sockman et al. 2000). In female turkeys, *Meleagris gallopavo*, and domestic hens, *Gallus gallus domesticus*, immunization against prolactin or the prolactin-releasing-factor vasoactive intestinal peptide (Sharp et al. 1998) inhibits incubation (Crisóstomo et al. 1997; Sharp 1997a; Crisóstomo et al. 1998), and treatment with exogenous prolactin promotes incubation if it is preceded by nesting behaviour (El Halawani et al. 1986; Sharp 1997b). Testosterone and prolactin in male kestrels may have functions similar to those described above, but this must be confirmed experimentally, either by administration of exogenous hormones or removal of endogenous hormones.

Once a causal relationship between testosterone and sexual behaviour and between prolactin and parental behaviour has been established for the male American kestrel, it would be interesting to investigate the extent to which seasonal change in these hormones influences a male's propensity to engage in aggressive or parental behaviour early and late in the season. Indeed, it is of interest that males whose mates were laying eggs (and therefore engaged in sexual activity) late in the breeding season had low levels of testosterone (Fig. 6b). In addition, these late-season males may have high prolactin levels prior to the onset of parental behaviour. Similarly, males displaying parental behaviour early in the season may have relatively low prolactin levels. This does not absolve the hormones from a causal role in the behaviours. A subtle but underappreciated aspect of behavioural endocrinology, articulated by Beach (1948), is that changing hormone concentrations do not necessarily induce behavioural changes but rather modulate the probability or intensity of a behaviour when the appropriate stimuli and conditions are present (Becker & Breedlove 1992). In a large, controlled experimental study, this modulatory influence of hormones may appear as a tightly coupled cause-and-effect relationship between the hormone and the behaviour. But, the behavioural change may at times occur in the absence of the hormonal change or the hormonal change may occur in the absence of the behavioural change. Paternal behaviour early in the season when prolactin levels are very low is feasible. Elevated prolactin may simply enhance the likelihood of the male partaking in parental care when conditions favour it (i.e. when eggs or young are in the nest) but not directly induce parental care. Similarly, elevated testosterone may enhance the likelihood of the male engaging in territoriality and courtship, but these behaviours may still occur when testosterone concentrations are low. In terms of adaptive significance, perhaps the seasonal change in prolactin tracks a seasonal change in the optimal resolution of the trade-off between paternal and nonpaternal behaviour following laying. Likewise, a seasonal decline in testosterone during sexual activity may track a seasonal decline in the need for territoriality and courtship.

It is also important to consider the fact that changes in hormone concentrations occurring outside the blood plasma may orchestrate behavioural changes as well. Whereas we documented changing plasma concentrations, the vertebrate brain can produce and concentrate testosterone (Compagnone & Mellon 2000), as well as prolactin (Harlan et al. 1989). Not only might concentrations of hormones in the brain differ from those in the plasma, but the action of neural hormones may occur in the absence of changing plasma hormones (Schlinger et al. 2001). None the less, changing concentrations of plasma hormones is probably a significant factor regulating the transition from sexual to parental behaviour, and our findings that the passage of time drives some of these changes demonstrates a subtle and previously undetected level of hormonal modulation. Future studies may show that this leads to a seasonal modulation in behaviour.

By statistically removing the effects of the passage of time on hormonal changes, we demonstrate that the well-documented increase in prolactin concentrations associated with the transition from sexual to parental behaviour is, in the male kestrel's case, due to the passage of time. Additionally, the decline in testosterone concentrations that accompanies the transition from sexual to parental behaviour is modulated seasonally. It is possible that other phenomena attributed to environmental, behavioural, social or other variables also may be products of the association between such variables and the passage of time.

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