

Maternal androgens in the pied flycatcher: timing of breeding and within-female consistency

Michael Tobler · Martin Granbom · Maria I. Sandell

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Abstract Maternal hormones can have substantial phenotypic effects in the progeny of many vertebrates. It has been proposed that mothers adaptively adjust hormone levels experienced by particular young to optimize their reproductive output. In birds, systematic variation in egg hormone levels has been related to different female reproductive strategies. Because in many bird species prospects of the offspring change seasonally and with brood number, strategic adjustment of yolk androgen levels would be expected. To test this idea, we induced pied flycatcher (*Ficedula hypoleuca*) females to nest twice during the same season by removing their first clutches shortly after clutch completion. We collected eggs of first and replacement clutches to measure yolk concentrations of androstenedione (A4) and testosterone (T) and captured the females that laid these clutches for phenotypic measurements. Although average egg androgen levels were remarkably consistent within females, hormone patterns differed considerably between first and replacement clutches. Eggs of replacement clutches were heavier with larger yolks compared to first clutches, but they contained on average lower levels of androgens. Within clutches, androgen concentration increased over the laying sequence in the first clutch, but decreased or remained more constant over the laying sequence in the replacement clutch. Mean yolk T, but not A4 levels, were negatively associated with

laying date for both breeding attempts. Moreover, females in good body condition produced eggs containing lower levels of androgens than females in poor condition. Our results are consistent with the idea that differences in yolk androgen levels may be one mechanism underlying seasonal variation in reproductive success and it is possible that changes in egg androgen patterns may reflect a change in female reproductive strategy. High within-female consistency also highlights the possibility that there may be some underlying genetic variation in yolk androgen levels.

Keywords Maternal effects · Reproductive investment · Seasonal adjustment · Laying order · Clutch number

Introduction

According to life history theory, parents maximise their fitness by optimizing the development and quality of their offspring (Stearns 2003). Parents can influence their offspring's phenotype through reproductive decisions such as with whom to mate, where to nest or which and how many resources to invest. Mothers can modify offspring performance and quality by providing non-genetic maternal resources, such as water, RNA, antibodies or hormones (e.g. Mousseau and Fox 1998). In many vertebrates, maternally derived hormones play an important role in shaping offspring phenotype (Clark and Galef 1995; McCormick 1999; Uller and Olsson 2003; Groothuis et al. 2005b). It has been suggested that mothers might adaptively adjust hormone levels experienced by particular young and thereby optimize their reproductive output (Schwabl et al. 1997; Mousseau and

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M. Tobler (✉) · M. Granbom · M. I. Sandell
Department of Animal Ecology, Lund University,
223 62 Lund, Sweden
e-mail: michael.tobler@zoekol.lu.se

Fox 1998). In birds, maternal androgens in the egg yolk promote postnatal growth (e.g. Schwabl 1996; Eising et al. 2001; Pilz et al. 2004; but see Sockman and Schwabl 2000) and chick competitiveness (e.g. Schwabl 1996; Eising and Groothuis 2003), but also suppress immune function (Groothuis et al. 2005a; Müller et al. 2005; but see Tschirren et al. 2005). Differences in egg androgen concentration both within and among clutches may therefore have important consequences for offspring survival and brood size. Specific within-clutch androgen patterns, such as increasing or decreasing androgen levels with laying order, may reduce or enhance size hierarchies and competitive interactions among siblings and, hence, reflect specific female strategies to avoid or to facilitate brood reduction (sensu Schwabl et al. 1997). Similarly, among-clutch variation in yolk androgens may reflect adaptive female investment. Several studies suggest that the transfer of maternal androgens is influenced by the expected reproductive value of the offspring. Higher amounts of androgens are found in eggs of females that are mated to more attractive males (Gil et al. 1999, 2004, 2005; Tanvez et al. 2004; but see Mazuc et al. 2003), which are likely to sire offspring of better genetic quality or provide other benefits in terms of territory quality or parental care. Correlative studies also found a positive association between yolk androgen levels and female quality (Pilz et al. 2003; Gil et al. 2005). That high levels of yolk androgens are associated with high parental quality suggests that these hormones entail some cost (e.g. reduced immune function) which is either incurred by the offspring, the mother or both.

Several studies on altricial birds have documented a seasonal change in female reproductive tactics (e.g. Slagsvold and Lifjeld 1988; Qvarnström et al. 2000; Nilsson 2000; Lifjeld et al. 2005). Such changes are generally interpreted as an adaptive response to seasonally changing prospects of the offspring (Drent and Daan 1980; Daan et al. 1990; Rowe et al. 1994). For example, seasonal decline in clutch size, which is common in many bird species, may be a strategic adjustment to declining food supply late during the breeding season and to seasonally declining survival prospects of fledglings (e.g. Slagsvold and Lifjeld 1988; Winkler and Allen 1996; Nilsson 2000; Lifjeld et al. 2005). Similarly, hatching asynchrony, which creates a size hierarchy and, hence, competitive asynchrony among siblings often increases with the progress of the breeding season (e.g. Slagsvold 1986; Veiga 1992; Nilsson 2000). This has been interpreted as a potential female strategy to facilitate brood reduction in late clutches (e.g. Lack 1966; Magrath 1989; Amundsen and Slagsvold 1991). Given that maternal yolk hormones are assumed to play an important role in reproductive optimization, modulating

competitiveness among siblings, adjustment of egg hormone levels to seasonally changing prospects of offspring would be expected. It is possible that seasonal changes in the concentration of maternal egg androgens contribute to the seasonal variation in hatching asynchrony or fledging success which has been observed in many bird species. So far, seasonal decline in mean yolk androgen levels in wild birds has been attributed to variation in female quality (Pilz et al. 2003; Gil et al. 2005). However, in a recent study on barn swallows, Gil et al. (2005) found that females laid eggs with significantly less androgens in second than in first clutches. This reduction in yolk androgen levels could be an adaptive response to reduced reproductive value of late clutches. Furthermore, a study on black-headed gulls suggests that also within-clutch patterns of androgens may change seasonally, because yolk testosterone (T) increased more steeply over the laying order in late compared to early clutches (Müller et al. 2004).

The aim of this study was to experimentally investigate whether there is systematic seasonal variation in yolk androgen levels in the pied flycatcher (*Ficedula hypoleuca*) and if any differences found may be attributed to strategic reproductive decisions by females. We induced female pied flycatchers to re-nest by removing their first clutches, which allowed us to examine individual androgen patterns in two successive clutches laid at different dates of the season. Since hatching asynchrony usually increases and fledging success declines with the progress of the breeding season in this species (Lundberg and Alatalo 1992), we predicted that females would transfer relatively lower levels of androgens in the late-laid eggs of their replacement clutch. We also evaluated whether patterns of yolk androgen variation are related to seasonal variation in egg characteristics (egg mass, yolk mass) and female quality covariates (clutch size, laying initiation date, inter-clutch interval, female condition). In addition, collection of two successive clutches also allowed us to estimate within-female consistency of yolk androgen levels, an aspect which has received little attention so far. Such estimates are interesting as they may give some indication about whether there may be some underlying genetic variation in yolk hormone levels of individual females and, hence, whether they may be subject to natural selection.

Material and methods

Study species and study area

The pied flycatcher is an insectivorous passerine, which breeds in natural cavities and nest boxes throughout

Europe. It overwinters in tropical West Africa and arrives at the study area by the end of April. It usually lays a single clutch of four to eight eggs, but can re-lay within 1–2 weeks in the case of clutch loss. Predation on nests in natural cavities and in nest boxes is common. Nilsson (1984) reports predation rates of 5–25% for pied flycatcher nests in another population in southern Sweden. The pied flycatcher normally has a semi-asynchronous hatching, with a hatching span of 1–2 days (Lundberg and Alatalo 1992). The study was conducted in Vombs Fure (ca. 55°39'N, 13°34'E), in southernmost Sweden. Vombs Fure is a pine plantation of ca. 225 ha and medium age. Because there are only few natural cavities, most of the nesting takes place in the ca. 130 nest boxes erected in the area.

General field methods

From 1 May to 13 June 2004 all nest boxes were visited at least every 3 days to check for any sign of nest building. When a nest was about two-thirds complete it was monitored daily to determine laying order of the eggs. Each egg in a clutch was marked individually with a non-toxic pen on the day of laying. On nine occasions, when the female sat inside the controlled nest box, the egg laid that day was not marked. This resulted in two unmarked eggs the next day. In these cases, eggs received an average egg number (e.g. 2.5 in a case where the second and third egg were unmarked). To avoid embryonic development due to onset of incubation, eggs were collected and replaced with plastic ones as soon as a clutch contained four eggs. All later eggs were collected and replaced daily. Although females may potentially have started to incubate on the first three eggs, we found no sign of embryo development in any of the eggs and eggs were never warm at the time of collection. There was no evidence of egg dumping as we never found more than one newly laid egg per day in a nest box. Moreover, intra-specific brood parasitism is assumed to be very low in this species (Lundberg and Alatalo 1992). After collection, eggs were weighted to the nearest 0.001 g and then frozen at -50°C for further yolk and androgen analysis. A clutch was assumed to be complete when no new egg was laid for a period of 3 days and the female had started incubating. On day 3 or 4 after clutch completion, the female was captured while incubating and all plastic eggs were removed. At the time of capture, we marked all females ($n = 25$) with an aluminium ring and took a measure of weight and tarsus length. Blood samples were also taken from the females for the purpose of another study. One female escaped before we could measure its weight. Female condition was calculated as

the residual of the regression of body mass on tarsus length; the residuals were calculated separately for the first and the second breeding attempt. Replacement nests of as many females as possible were located and laying order of the eggs was determined as described above. The first female found re-laying was the one who had initiated the earliest clutch in our population. The last “first clutch” used in this study was initiated the day the first female started re-laying (or 16 days after the first egg was laid at Vombs Fure). Hence, we assume that none of the females used in this study had made an earlier breeding attempt outside the study area. Out of 20 females found re-laying, we were able to recapture 17 for weighing and blood sampling after completion of the replacement clutch.

Hormone assays

Shell, albumin and yolk were separated during thawing of the egg to allow for analyses of albumin and yolk mass. After yolk mass was measured, the yolk was homogenized and a small sample (approximately 25 mg) was used for hormone analysis. Individual samples were mixed with 500 μl distilled water in 1.5-ml Eppendorf tubes using a Vortex mixer. Homogenization was facilitated by the addition of a few glass beads in each tube (Schwabl 1993).

We analysed hormone levels in eggs from 20 individual females which laid a first and a replacement clutch. However, two full first and replacement clutches were accidentally misplaced and could not be used for hormone analysis. All samples were analysed for the presence of androstenedione (A4) and T using radioimmunoassay kits from Diagnostic System Laboratories (DSL). Crossreactivity with other androgens are low in both kits (less than 1% in A4 kits, DSL-3800 and less than 6% in testosterone kits, DSL-4000). Tritiated forms of each steroid were added to each sample for calculations of recovery percentages following extraction (approximately 2,000 c.p.m., Perker Elmer Life Sciences). Steroids were extracted twice with 3 ml petroleum and diethyl ether (30:70%), following a precipitation with 95% ethanol to remove excess proteins and lipids. After extraction, samples were resuspended in buffer (PBSG). Hormones were measured with coated-tube radioimmunoassay kits for A4 (DSL-3800) and T (DSL-4000). Each sample was run in duplicates and mean values were compared with a standard curve. The samples were analysed in two assays with all eggs belonging to a female within the same assay. Intra-assay variation was 6.1 and 8.0% for A4, and 4.3 and 5.5% for T; inter-assay variation was 4.5% for A4 and 1.3% for T.

Statistics

Statistical tests were conducted with SAS System for Windows, version 9.1. Egg and yolk mass, as well as yolk androgen levels were log-transformed to normalize the residuals. We used general linear mixed models (PROC MIXED; Littell et al. 2004) for analyses which involved several clutches from the same female and/or several eggs from the same clutch. Clutch number (first or replacement clutch) was used as a fixed factor and laying order as a covariate. Female identity and its interactions with the fixed factor and covariate were included as random factors in the model. The random effects were estimated with the likelihood ratio test (Littell et al. 2004). In analyses in which we tested the relationship between maternal characteristics and egg parameters, we used clutch means (e.g. mean A4 concentration) as dependent variables. In these models, female identity was included as a random factor, clutch number as a fixed factor and the characteristic of interest (clutch size, female condition, laying date, quadratic term of laying date) as a covariate. Satterthwaite's correction was used to calculate the denominator df in all mixed models. The full model, which was used to analyse variation in yolk mass did not converge and we therefore excluded the random three-way interaction (female identity) \times (egg number) \times (clutch number) to achieve convergence. Due to this the Satterthwaite approximation produced a higher number of denominator df than in the other models. All interaction terms between fixed factors and covariates were initially included, but non-significant effects ($P > 0.1$) were sequentially removed from the models. When relating interbrood interval to laying date, we used non-parametric Spearman rank correlation because interbrood interval could not be normalized. Repeatability of mean egg mass, mean yolk mass and mean androgen levels between first and

replacement clutch was calculated following Lessells and Boag (1987).

Results

Egg characteristics

Egg mass differed significantly among females ($\chi^2 = 14.4$, $df = 1$, $P < 0.0001$). Eggs of first clutches were significantly lighter on average than those of replacement clutches (Table 1). Both in first and replacement clutches, egg mass increased significantly over the laying sequence ($F_{1,26.5} = 25.88$, $P < 0.0001$). The slope did not differ between the two clutches (interaction between clutch number and laying order: $F_{1,70.5} = 0.36$, $P = 0.55$). The increase of egg mass between the first and the last egg was +4.5% in the first clutch and +5.1% in the replacement clutch.

Yolk mass differed significantly among females ($\chi^2 = 6.0$, $df = 1$, $P = 0.007$). Yolks of first clutches weighted significantly less on average than yolks of replacement clutches (Table 1). However, the difference in yolk mass between first and replacement clutch was influenced by laying order. There was a significant interaction between clutch number and egg number ($F_{1,227} = 7.46$, $P = 0.007$), which was due to a decrease in yolk mass over the laying sequence in the first clutch and an increase over the laying sequence in the replacement clutch. The relative change in yolk mass between the first and the last egg was -4.0% in the first clutch and +1.9% in the replacement clutch.

Because yolk mass changed systematically over the laying sequence, analyses were performed both on androgen concentration (pg androgen per mg yolk) as well as total androgen content (ng androgen per yolk). Unless the results are different, however, we only report analyses for yolk androgen concentration. Eggs

Table 1 Mean \pm SE and range of clutch size, egg mass, yolk mass, androstenedione (A4) and testosterone (T) content and concentration for first and replacement clutches of female pied flycatchers

	First clutch		Replacement clutch		Denominator df^a	F-statistic ^b
	Mean \pm SE	Range	Mean \pm SE	Range		
Clutch size	7.0 \pm 0.16	6–9	5.70 \pm 0.22	5–9	21.2	54.63**
Mean egg mass (g)	1.62 \pm 0.02	1.48–1.75	1.70 \pm 0.02	1.57–1.88	19.5	32.76**
Mean yolk mass (mg)	345 \pm 5	304–389	368 \pm 7	327–449	21.4	21.40*
A4 content (ng/yolk)	38.1 \pm 2.8	12.8–63.2	33.0 \pm 3.7	8.2–66.1	15.2	15.99*
A4 concentration (pg/mg yolk)	110.6 \pm 8.1	34.6–180.6	89.7 \pm 9.8	22.7 \pm 180.4	15.3	21.25**
T content (ng/yolk)	8.6 \pm 1.0	3.3–20.4	6.2 \pm 0.6	2.3–10.7	15.2	37.91**
T concentration (pg/mg yolk)	25.1 \pm 3.2	9.7–67.2	17.1 \pm 1.8	6.2–32.1	15.1	43.08**

* $P < 0.005$, ** $P < 0.0005$

^a Denominator df ; nominator df is 1 in all cases

^b Mixed models

of pied flycatchers contained about 5 times higher A4 levels than T levels (Table 1). Androgen concentration differed significantly among females (A4, $\chi^2 = 7.4$, $df = 1$, $P = 0.0003$; T, $\chi^2 = 16.1$, $df = 1$, $P < 0.0001$). Concentrations of A4 and T were highly correlated ($F_{1,215} = 30.8$, $P < 0.0001$). The mean androgen concentration in eggs of first clutches was significantly higher than that of replacement clutches (Table 1). Within-clutch androgen patterns differed also between the two clutches. There was a significant interaction between clutch number and laying order for A4 concentration ($F_{1,37.7} = 4.68$, $P = 0.037$; Fig. 1). A4 concentration increased with laying order in the first clutch, but decreased in the replacement clutch. The relative change in A4 concentration between the first and the last egg was +14.7% in the first clutch and -2.76% in the replacement clutch. A similar change in within-clutch pattern was found for T concentration although the interaction was not quite significant ($F_{1,46.1} = 3.31$, $P = 0.075$; Fig. 1). The increase in T concentration between the first and the last egg was 28.2% in the first clutch, but only 13.2% in the replacement clutch. Taking within-clutch variation of yolk mass into account by performing the same analysis on total A4 and T content, neither of the two interactions was significant (A4, $F_{1,41.3} = 2.49$, $P = 0.12$; T, $F_{1,45.5} = 1.58$, $P = 0.21$). Moreover, when using content in the analysis there was no overall pattern with respect to laying order for either of the two androgens (A4, $F_{1,22.1} = 0.14$, $P = 0.72$; T, $F_{1,27.6} = 1.19$, $P = 0.29$). The discrepancy in within-clutch pattern between total content and concentration is a consequence of yolk mass varying over the laying sequence in a manner that is opposite to that of the androgens.

Female quality covariates

First clutches were significantly larger than replacement clutches (Table 1). Neither mean egg or yolk mass nor the mean yolk concentrations of A4 or T were significantly related to clutch size ($F < 0.9$, $df > 1.25$, $P > 0.30$).

Mean egg mass and yolk mass were not significantly related to female body condition (main effects and interactions with clutch number, $F < 0.2$, $df > 1,29$, $P > 0.60$). In contrast, mean yolk A4 concentration was significantly negatively related to female body condition ($F_{1,28.2} = 5.00$, $P = 0.033$; Fig. 2). The relationship was the same in both breeding attempts, but A4 concentration tended to be lower in replacement clutches for a given value of condition (effect of clutch number, $F_{1,18.4} = 4.30$, $P = 0.052$; Fig. 2). A similar relationship was found for mean yolk T concentration and female

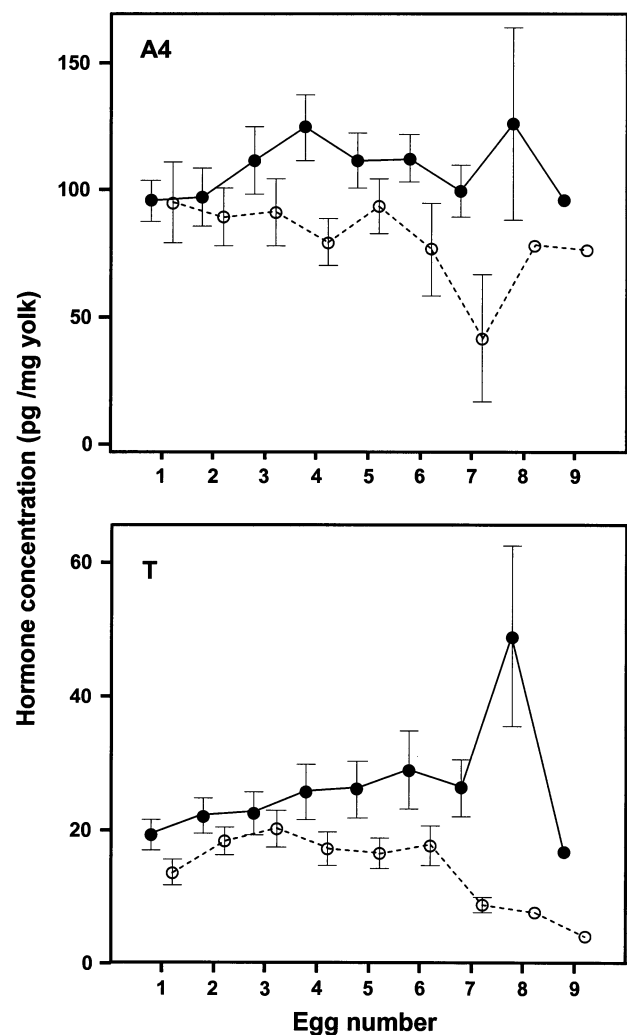


Fig. 1 Mean yolk concentration (± 1 SE) of androstenedione (A4) and testosterone (T) in relation to laying order of the eggs in first (filled circles) and replacement clutches (open circles). Egg number 1 is the first laid egg in a clutch. There were 18 first and 18 replacement clutches. Eggs for which exact laying date could not be determined (see text) are not represented in the figure. Statistical analyses included all eggs

body condition although it was not quite significant ($F_{1,22.3} = 3.45$, $P = 0.076$; Fig. 2). Instead, there was a strong effect of clutch number ($F_{1,16.1} = 10.81$, $P = 0.005$). There was no significant interaction between female body condition and clutch number for either of the two androgens ($F < 0.06$, $df > 1,11.8$, $P > 0.80$). Changes in female body condition between the first and the second breeding attempt were not related to mean egg mass, mean yolk mass or mean androgen levels in the replacement clutch ($F < 2.5$, $df > 1,12$, $P > 0.13$).

Mean egg and yolk mass did not vary in relation to laying initiation date or the quadratic term of laying initiation date ($F < 2.5$, $df > 1,28$, $P > 0.10$). There was

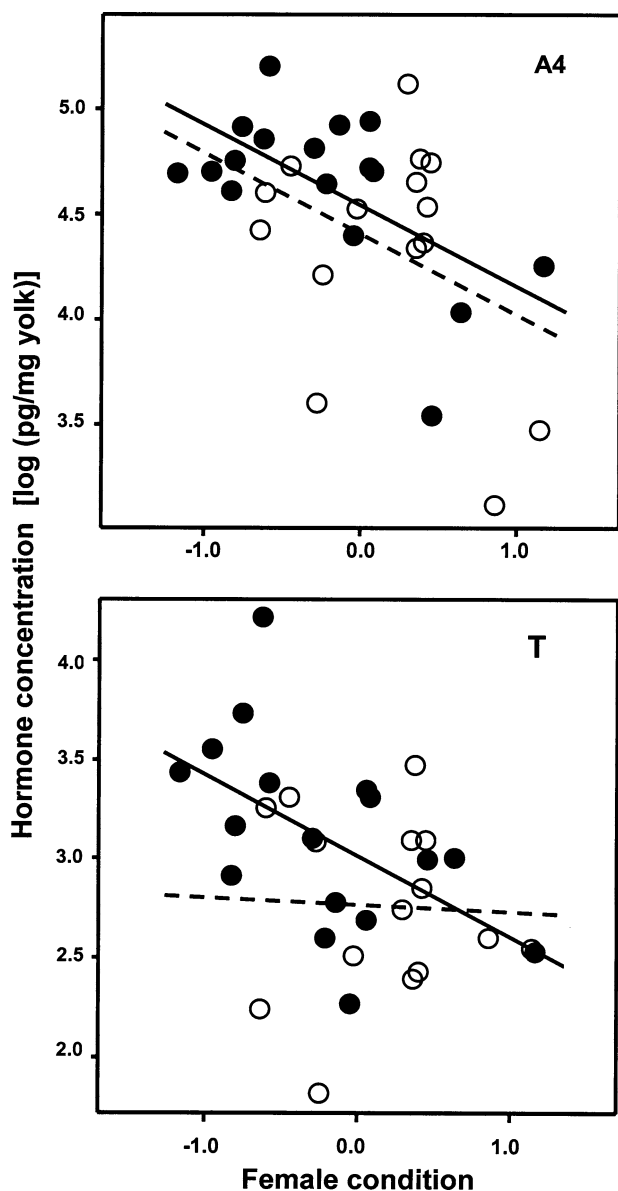


Fig. 2 Relationship of female body condition (the residual of the regression of body mass on tarsus length) with concentration of yolk A4 and yolk T. Mean concentrations of first clutches (filled circles; $n = 17$) and replacement clutches (open circles; $n = 15$) are depicted. Samples sizes differ because we were unable to measure body mass for one female at the first breeding attempt and three females at the second breeding attempt. For abbreviations, see Fig. 1

also no significant association between mean yolk A4 concentration and laying initiation date ($F_{1,26.5} = 0.01$, $P = 0.92$) or its quadratic term ($F_{1,31.4} = 0.01$, $P = 0.94$) (Fig. 3). For mean yolk T concentration, there was no significant relationship with laying initiation date ($F_{1,28.5} = 1.55$, $P = 0.22$), but instead with the quadratic term of laying initiation date ($F_{1,32.9} = 4.56$, $P = 0.04$) (Fig. 3). Mean T concentration decreased more strongly towards the end of the study period. Clutch

number did not significantly affect any of the relationships between egg mass, yolk mass or androgen levels and laying initiation date ($F < 1.42$, $df > 1,28$, $P > 0.24$) and there was also no significant interaction between clutch number and laying initiation date or its quadratic term ($F < 2.1$, $df > 1,15.7$, $P > 0.18$). The length of the interbrood interval (i.e. the number of days between the last egg of the first clutch and the first egg of the second clutch) ranged from 8 to 11 days and was strongly negatively correlated with laying date (Spearman rank correlation, $r = -0.76$, $n = 20$, $P = 0.0001$). This means that females initiating their first brood late in the breeding season re-laid more quickly than females initiating their clutch early in the breeding season. However, length of the interbrood interval was not related to concentration of yolk androgens in the replacement clutch (A4, $F_{1,16} = 0.85$, $P = 0.37$; T, $F_{1,16} = 1.83$, $P = 0.19$).

Repeatability of egg parameters

Mean egg mass was significantly repeatable between the first and the replacement clutch ($R = 0.58$, $F_{19,20} = 3.73$, $P = 0.003$). Repeatability of mean yolk mass was considerably lower than that of egg mass, but still significant ($R = 0.39$, $F_{19,20} = 2.28$, $P = 0.037$). Repeatability of mean androgen concentration between first and replacement clutch was highly significant (A4, $R = 0.58$, $F_{15,16} = 3.80$, $P = 0.006$; T, $R = 0.56$, $F_{15,16} = 3.52$, $P = 0.009$; Fig. 4).

Discussion

Yolk androgen levels in first and replacement clutches

Eggs collected from two successive clutches laid within the same breeding season differed markedly in androgen concentration. Within clutches, yolk androgen concentration increased over the laying sequence in the first clutch, but decreased or remained more constant over the laying sequence in the replacement clutch. Moreover, eggs of first clutches contained on average significantly more androgens than replacement clutches. Below, we discuss the potential causes and consequences of this difference in yolk androgen levels.

As predicted, relatively lower levels of A4 and T were found in late-laid eggs of replacement clutches compared to first clutches. This change in within-clutch androgen pattern between the two breeding attempts may reflect an adaptive adjustment of the female breeding strategy in relation to season. Given that yolk androgens boost growth and enhance competitive ability of

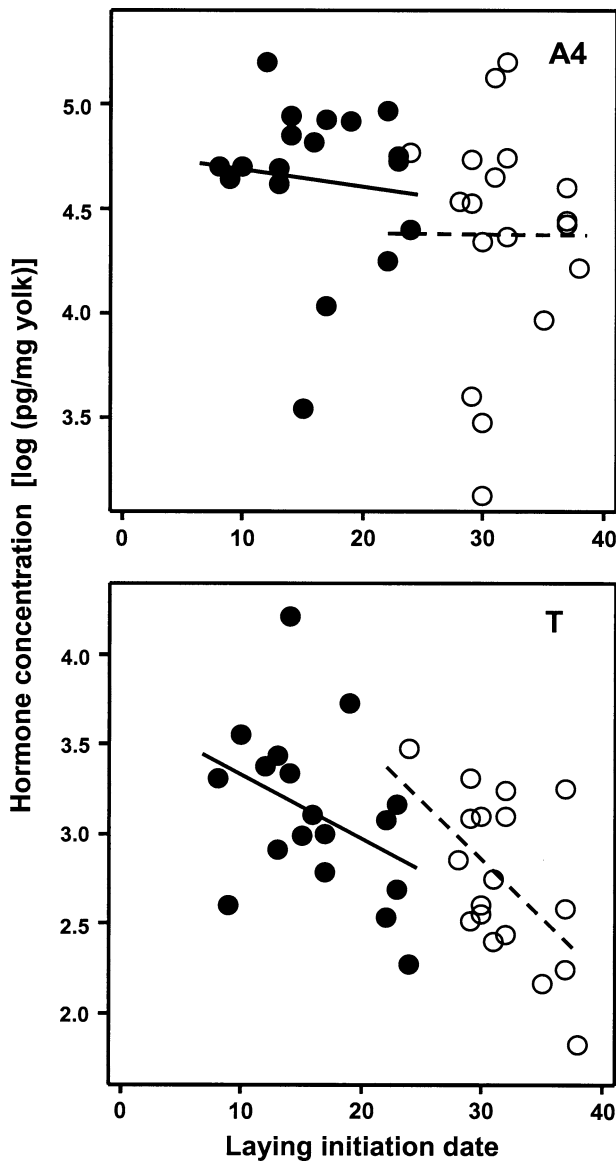


Fig. 3 Relationship between the date a female initiated a clutch and the mean concentrations of yolk A4 and T. *Filled circles* depict first clutches ($n = 18$) and *open circles* replacement clutches ($n = 18$). Laying initiation date 1 = May 1. For abbreviations, see Fig. 1

offspring (see [Introduction](#)), size hierarchies resulting from hatching asynchrony would be expected to be reduced in first clutches, but enhanced in replacement clutches. In other words, females may adopt a brood survival strategy in their first breeding attempt, but a brood reduction strategy in their second breeding attempt. Such a seasonal change in breeding strategy may be beneficial as food availability in temperate areas usually becomes less predictable late during the breeding season (Lack 1966; Perrins 1970) and brood reduction at times when food availability is more variable may enhance parental fitness (Lack 1966; Slagsvold

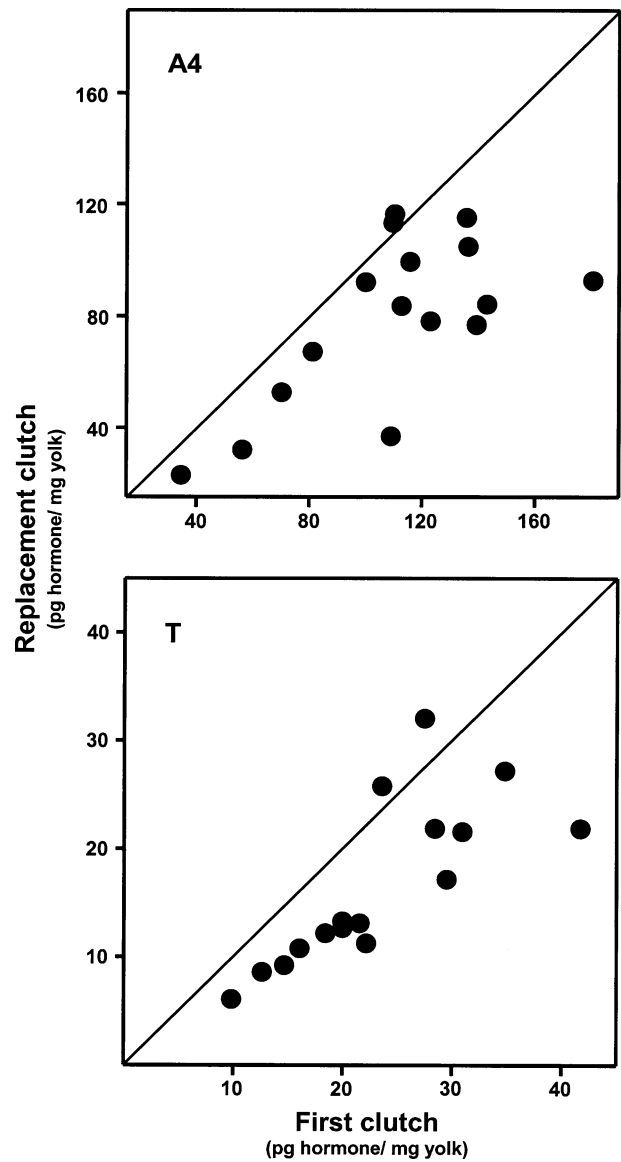


Fig. 4 Relationship of A4 and T concentration between first and replacement clutch. Points would be on the diagonal line if the androgen contents were identical in the first and replacement clutch ($n = 16$). For abbreviations, see Fig. 1

1986; Magrath 1989). This may be especially so for a species like the pied flycatcher, which is a long-distant migrant that undergoes a complete moult before the onset of autumn migration. Several studies indicate that adult pied flycatchers trade off the onset of their moult against the time and energy needed for their brood (Hemborg and Lundberg 1998; Siikamaki 1998; Hemborg 1999). Unfortunately, we have no data available on the hatching spread in our study population and, thus, we do not know whether hatching asynchrony would indeed be higher in replacement clutches. A study by Slagsvold and Lifjed (1989) in a Norwegian population found no difference in estimated hatching

spread between initial and repeat nestings. However, this finding contrasts with studies on the same Norwegian population and other populations in Sweden, which show that hatching asynchrony increases with laying date (Slagsvold 1986; Lundberg and Alatalo 1992). If facilitated brood reduction in late clutches would be adaptive, then a decrease in egg mass with the laying sequence to further increase the disadvantage of the last offspring would be expected (Clark and Wilson 1981). In our study, egg mass increased over the laying sequence also in the replacement clutch, which works against the brood reduction hypothesis and does not fit with the observed within-clutch pattern of androgen concentration. However, Krist et al. (2004) recently found that intra-clutch egg-size variation had no influence on offspring performance in the closely related collared flycatcher and therefore questioned its adaptive significance.

It is important to note that within-clutch androgen patterns with respect to laying sequence were only found for androgen concentration but not for total androgen content. This means that embryos from different eggs within a clutch are exposed to different androgen concentrations, but the total amount available during early development is similar. This result contrasts with studies on other altricial passerines in which no such difference between androgen concentration and content was found (Pilz et al. 2003; Tschirren et al. 2004).

Apart from the difference in within-clutch androgen patterns, eggs of first clutches contained on average more androgens than replacement clutches. Differences in mean androgen levels between first and replacement clutches may be a consequence of seasonal variation in brood sex ratio, which has been reported for several bird species (e.g. Daan et al. 1996; Lessells et al. 1996; Rosivall et al. 2004). Although maternal yolk androgens do not seem to influence genetic sex determination in birds (Pilz et al. 2005; see also Groothuis et al. 2005b for a review on this topic), male and female eggs may differ in the amount of yolk androgens they contain (Müller et al. 2002). As we did not determine the sex of the collected eggs, we cannot test the hypothesis that differences in mean androgen levels between first and replacement clutches are related to differences in brood sex ratio. However, a study on the closely related collared flycatcher (*Ficedula albicollis*), did not reveal sex-specific differences in nestling mortality and recruitment, even when the nestlings were raised under poor conditions (Sheldon et al. 1998). Seasonal changes in brood sex ratio are, thus, less likely to be favoured by natural selection in this species.

An alternative explanation for the differences in mean yolk androgen levels between first and replacement clutches may be that the transfer of maternal androgens simply is an inevitable consequence of the female state. Lower yolk androgen levels in replacement clutches may be associated with reduced androgen levels in the female circulation at the time of yolk formation. This hypothesis is supported by a study of Wingfield and Farner (1979), which shows that females of the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) have lower circulating androgen levels during yolk deposition when producing their replacement clutch than when producing their first clutch. Such seasonal changes in female blood hormone levels may be the consequence of an altered female reproductive state or they may be related to changes in the intensity of social interactions (see Ketterson et al. 2005 for a recent review). Notably, however, to date there is little direct evidence that yolk hormone levels are associated with maternal blood levels of these hormones (see Groothuis et al. 2005b for a review).

In sum, it is possible that changes in within-clutch androgen patterns reflect an adaptive female adjustment to facilitate brood reduction in experimentally delayed clutches. The functional significance of the decrease in mean androgen levels between first and replacement clutches is unclear, however.

Female covariates in repeated clutches

Few studies have examined the relationship between yolk androgen levels, clutch size and laying date. Pilz et al. (2003) found that in the European starling (*Sturnus vulgaris*) androgen levels were positively related to clutch size, but negatively to laying date. Very similar results were obtained in a recent study on barn swallows (*Hirundo rustica*) (Gil et al. 2005). The existence of such relationships has been interpreted as evidence for the hypothesis that high androgen levels represent a costly maternal investment. Early breeders producing large clutches usually have a higher reproductive success and are of higher parental quality than late breeders with small clutches (e.g. Drent and Daan 1980; Daan et al. 1990; Rowe et al. 1994; Price 1998). In our study, mean yolk androgen levels were not significantly related to clutch size, but we found a significant negative relationship between mean yolk T levels and the quadratic term of laying initiation date. Although this result is consistent with the previous studies, our data do not allow us to distinguish whether the decrease in mean androgen levels is an effect of laying date per se or an effect of the experimental delay in breeding.

We found a negative relationship between yolk androgen levels and female body condition. Body condition was measured after clutch completion, i.e. after the females had invested in egg production. It is possible that higher androgen levels are associated with higher investment in egg production. Alternatively, females of higher quality may be able to breed at a lower condition and transfer more androgens into their eggs. However, as this is a correlative study, it is not possible to conclude that low condition is the consequence rather than cause of increased androgen transfer.

Within-female consistency of yolk androgen levels

Collection of two successive clutches allowed us to estimate within-female consistency of yolk androgen levels. In accordance with previous studies of other species (e.g. Reed and Vleck 2001; Pilz et al. 2003; Tschirren et al. 2004), we found large inter-female variation in yolk androgen levels. Interestingly, mean yolk androgen levels were remarkably consistent within individual females between the two breeding attempts. Repeatability for mean yolk A4 and T (0.58 and 0.56) levels was as high as the one for mean egg mass, which is generally high among birds (Christians 2002). Notably, repeatability of yolk androgen levels in our study was much higher compared to the one reported for yolk A4 in a recent study on barn swallows (Gil et al. 2005). Gil et al. (2005) found a significant within-female repeatability for first and second clutches in yolk A4 concentration of 0.22. The discrepancy between the two studies may partly be due to the fact that our repeatability measures are based on mean androgen levels whereas the measures in Gil et al.'s study are based on the androgen level from the first or second egg only. Indeed, repeatability estimates for A4 and T content in pied flycatchers were much lower and not quite as significant when based on androgen levels of the first-laid egg only (A4, $R = 0.45$, $F_{15,16} = 2.66$, $P = 0.03$; T, $R = 0.40$, $F_{15,16} = 2.32$, $P = 0.052$). Furthermore, it is possible that our repeatability estimates are somewhat higher because time until the production of the second clutch was much shorter in our study. We removed all eggs when the clutch was complete and pied flycatcher females started to re-lay within less than 2 weeks. Female barn swallows in Gil et al.'s (2005) study, on the other hand, were allowed to keep their eggs and to raise their offspring. Hence, the time between two clutches was probably more than 1 month. Nevertheless, the results suggest that mean androgen level may be a characteristic of individual females and that there may be some underlying genetic variation in yolk androgen levels.

Conclusions

Our results are consistent with the idea that differences in yolk androgen levels may be one mechanism underlying seasonal variation in reproductive success and it is possible that the change in within-clutch androgen patterns reflects a shift in female reproductive tactic. However, at this stage proximate constraints that affect androgen transfer in egg-laying females cannot be excluded. Our study suggests further that yolk androgen levels may be a female characteristic, which may have a heritable component. Future studies will have to address whether androgen levels are consistent over several breeding seasons or generations, respectively.

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