Yolk androgens reduce offspring survival

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Females may favour some offspring over others by differential deposition of yolk hormones. In American kestrels (*Falco sparverius*), we found that yolks of eggs laid late in the sequence of a clutch had more testosterone (T) and androstenedione (A₄) than yolks of first-laid eggs. To investigate the effects of these yolk androgens on nestling 'fitness', we injected both T and A₄ into the yolks of first-laid eggs and compared their hatching time, nestling growth and nestling survival with those of first-laid eggs in which we injected vehicle as a control. Compared to controls, injection of T and A₄ at a dose intended to increase their levels to those of later-laid eggs delayed hatching and reduced nestling growth and survival rates. Yolk androgen treatment of egg 1 had no effect on survival of siblings hatching from subsequently laid eggs. The adverse actions of yolk androgen treatment in the kestrel are in contrast to the favourable actions of yolk T treatment found previously in canaries (*Serinus canaria*). Additional studies are necessary in order to determine whether the deposition of yolk androgens is an adaptive form of parental favouritism or an adverse by-product of endocrine processes during egg formation. Despite its adaptive significance, such 'transgenerational' effects of steroid hormones may have helped to evolutionarily shape the hormonal mechanisms regulating reproduction.

Keywords: avian brood reduction; *Falco sparverius*; hatching asynchrony; maternal androgens; parental favouritism; parent-offspring conflict

1. INTRODUCTION

Parental overproduction of offspring is ubiquitous (Kozłowski & Stearns 1989) but often rectified when parents foster sibling competition that leads to brood reduction (Forbes & Mock 1998; Mock & Parker 1998). Sibling competition elevates the average quality of offspring because it promotes the survival of those that are the most competitive (Forbes & Mock 1998). However, parents (Hussell 1972) and their young (Hamilton 1964; Hahn 1981) may incur substantial energetic costs from competitions among evenly matched siblings (Forbes & Mock 1998). Thus, parents favour or handicap some offspring, tilting the 'playing field' and reducing the costs of sibling competition (Forbes & Mock 1998).

Evolution has produced several mechanisms for parental favouritism. Prominent among them is the production of sibling age hierarchies when parent birds hatch some eggs before others in a process known as hatching asynchrony (reviewed in Magrath 1990). These age differences may lead to competitive and developmental hierarchies among siblings (Mead & Morton 1985; Evans 1996; Nilsson & Svensson 1996; Stoleson & Beissinger 1997). The older and typically larger siblings may dominate the brood from its inception and, over the course of competition for parentally provided resources, further secure their dominance through a positive feedback loop between competitive ability and resource acquisition.

The discovery of maternally derived hormones in vertebrate eggs (Dickhoff *et al.* 1990; Feist *et al.* 1990; Prati *et al.* 1992; Conley *et al.* 1997; Janzen *et al.* 1998) raised the possibility that parental favouritism may also take the

form of differential hormone allocation. In laboratory canaries (*Serinus canaria*) with *ad libitum* food access, yolk testosterone (T) does not appear to influence embryonic development but does enhance nestling begging vigour and growth rates in broods in which hatching was experimentally synchronized (Schwabl 1996a). Depending on species, the concentrations of androgens deposited in avian egg yolks may increase (Schwabl 1993; Lipar *et al.* 1999) or decrease (Schwabl *et al.* 1997; Gil *et al.* 1999) with the order in which eggs of a clutch are laid (laying sequence). These findings led to the hypothesis of maternal favouritism by differential androgen allocation (Schwabl *et al.* 1997), where yolk androgens were seen as beneficial to offspring. However, additional studies were necessary in order to determine the effects of yolk androgens in other species and under different ecological conditions.

Sibling American kestrels (*Falco sparverius*) show pronounced developmental hierarchies which may give rise to brood reduction (Wiebe & Bortolotti 1995; K. W. Sockman, personal observation). As in other species, incubation usually begins before clutch completion and probably influences hatching asynchrony and the subsequent sibling hierarchy (Bortolotti & Wiebe 1993). However, as the presence of yolk androgens in other species suggests, female kestrels may use factors in addition to early incubation onset to discriminate among offspring and modify the developmental disparity among siblings. We measured and manipulated concentrations of T and androstenedione (A₄) in yolks of American kestrel eggs in order to determine whether the effects of yolk androgens on embryonic and nestling development in the domesticated canary (Schwabl 1996a) apply to free-living birds potentially contending with limited food resources and whether differential allocation of yolk androgens might facilitate brood reduction.

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2. METHODS

(a) General field procedures and species

We hung nest-boxes (inside, 17.4 cm width $\times$ 16.3 cm depth $\times$ 33.8 cm height) at *ca.* 0.5 km intervals along roadsides within a 35 km radius of the Washington State University campus (Pullman, Washington, USA, 46°44' N, 117°10' W). Originally a typical temperate steppe ecoregion (Bailey 1998), this area has since undergone widespread conversion mainly to wheat, barley and pea farming. We hung boxes at *ca.* 2–3 m height on trees or posts and provided pine shavings in each to protect eggs from the hard floor. We checked the boxes every three to four days for signs of occupancy. During laying, we checked the boxes every one to three days and marked new eggs. American kestrels usually lay every two days until the clutch is complete at four to six eggs, but laying intervals of one and three days sometimes occur. Incubation and nestling periods, each *ca.* 30 days, follow, after which nestlings fledge (Balgooyen 1976; Sockman & Schwabl 1998).

(b) Yolk collection and androgen measurements

In Spring 1997 and 1998, we collected and froze either yolk samples obtained by biopsy (Schwabl 1996b) or whole eggs within a few days of the eggs' being laid. We transferred thawed yolk (from biopsies) to extraction tubes along with an additional 0.5 ml of water. We separated yolks from frozen-thawed whole eggs, weighed and suspended them in water (1 ml per gram of yolk) and transferred 300–400 mg of the mixture to extraction tubes. We followed previously described procedures (Schwabl 1993) for the extraction, separation, partial purification and measurement of yolk T, A₄, dihydrotestosterone (DHT), 17 β -oestradiol (E₂) and corticosterone (B). DHT, E₂ and B concentrations were very low in most yolks, and we do not discuss these hormones further. We measured yolk T and A₄ in single radioimmunoassays with intra-assay coefficients of variation of 9.6 and 10.8%, respectively. Although some females laid up to six eggs, we obtained no yolk samples from egg 6 and only three samples from egg 5 of the laying sequence. In addition, most females in our study did not lay more than four eggs. Therefore, we limited our analyses to the first four eggs in the laying sequence (using only eggs of known laying order). Linear regressions indicated that time between laying and biopsy or egg collection did not affect T or A₄ concentrations in yolks (analysed separately by laying sequence) ($n \geq 9$ eggs, $F \leq 1.86$ and $p > 0.20$ for each analysis).

(c) Manipulation of yolk androgens

We measured the eggs' width and length in spring 1999, from which we calculated the eggs' volume (Hoyt 1979). Within a few days of clutch completion, we injected either 50 μ l sesame oil (control) or 0.1 μ g T and 4 μ g A₄ dissolved together in 50 μ l sesame oil (androgen treated) into the yolk of the first-laid egg in a clutch (Schwabl 1996a), thereby mimicking in egg 1 the high maternal doses found in later-laid eggs (see § 3). In order to calculate this dose we multiplied the mean yolk mass of collected eggs (see § 2(b)) by the difference between the mean concentration of each androgen found in egg 1 and the highest concentration of each found among eggs laid later in the laying sequence. Although we did not know the concentrations in individual eggs, this procedure should have elevated levels in egg 1 to the high levels found in eggs 3 and 4. We alternated clutches between androgen and control injections throughout the laying season.

(d) Quantification of hatching time, nestling growth and fledging

We visited the nests every one or two days, starting *ca.* 25 days after clutch completion (1999 season), until pipping occurred in eggs, at which point we visited the nests twice daily, once between 06.00 and 08.00 and once between 18.00 and 20.00. The frequency with which eggs hatch sometimes exceeds that with which we visited the nests, potentially making it difficult to assign a nestling to an egg. Therefore, we applied to the pipped area of the shell a small drop of food colouring which seeped into the egg through the pip. We alternated the colours applied among nests and eggs in case of potential effects of colour. We determined the eggs from which nestlings hatched based on colouring of the nestlings and clipped their talons for identification until they were old enough to be banded (15 days).

We considered nestlings found newly hatched within a single visit to have hatched simultaneously. Because of the frequency with which we visited the nests, we were only able to determine time of hatching to *ca.* 0.5 days. If, for example, we found egg 2 newly hatched one visit (*ca.* 12 h) after we had found egg 1 hatched, we considered egg 2 to have hatched 0.5 days after egg 1.

We measured nestling body mass and lengths of the tarsus, culmen and wing chord at every five days of age for days 0–25. We also measured the length of the tenth primary feather at ages ten, 15, 20 and 25 days and the lengths of the centre and outer rectrices at ages 15, 20 and 25 days. We defined nestling size as the first-axis factor scores of principal components analyses of the morphological measures (Rising & Somers 1989), not including body mass. Because these measures increase with age at different rates, we analysed them separately for each fifth day of age. Therefore, nestling size was relative to age and did not necessarily increase with age. We used all nestlings (not only those from egg 1) in the principal components analyses in order to generate the factor scores for egg 1 nestlings. We documented fledging by visiting the nests twice daily (between 06.00 and 08.00, and between 18.00 and 20.00) after the last 25-day-old nestling was measured and by noting when nestlings were no longer present in the nest-boxes. Analyses included only those eggs and nestlings in which we unequivocally knew the eggs' laying order and the eggs from which nestlings hatched.

3. RESULTS

(a) Concentrations of maternal yolk androgens

Yolk A₄ concentrations were substantially higher than yolk T concentrations (table 1). Concentrations of each androgen were significantly lower in first-laid than in later-laid eggs of the laying sequence. T and A₄ concentrations did not differ significantly among eggs 2–4 of the laying sequence. Neither T ($F_{1,19} = 0.78$ and $p > 0.20$) nor A₄ ($F_{1,19} = 2.25$ and $p = 0.15$) concentrations differed between 1997 and 1998, and the interaction between laying sequence (eggs 1–4) and year did not affect concentrations of either androgen (T, $F_{2,15} = 0.13$ and $p > 0.20$, and A₄, $F_{2,15} = 0.37$ and $p > 0.20$). The year and interaction terms were therefore removed from the statistical model described in table 1.

(b) Effects of yolk androgen treatment

Regardless of treatment, the injected egg hatched in all nests in which at least one egg hatched. One control and four androgen-treated nests were abandoned between hatching and fledging of the first nestlings. We do not

Table 1. Concentrations (picograms per milligram of yolk) of androgens in egg yolks of American kestrels

(We determined whether differences with respect to laying sequence in the clutch were significant using repeated-measures multiple analysis of variance (Wilks' λ , $F_{6,32} = 3.81$ and $p = 0.006$) where testosterone (T) and androstenedione (A_4) concentrations were each dependent variables measured repeatedly among eggs within a clutch. The values are means $\pm$ s.e.s with the numbers of eggs in parentheses. Values without a superscript in common were statistically different ($p < 0.05$) based on post-hoc linear contrasts. The F - and p -values are univariate statistics for each androgen.)

androgen	egg in laying sequence				$F_{3,17}$	p
	1	2	3	4		
T	7.75 $\pm$ 1.43 (14) ^a	15.86 $\pm$ 5.04 (9) ^b	16.78 $\pm$ 2.58 (11) ^b	14.42 $\pm$ 2.38 (8) ^b	5.36	0.009
A_4	178.3 $\pm$ 34.99 (14) ^a	335.13 $\pm$ 72.82 (9) ^b	631.36 $\pm$ 93.95 (11) ^b	611.24 $\pm$ 137.36 (8) ^b	7.58	0.002

Figure 1. Effect of yolk androgens on time to hatching in American kestrel eggs. We controlled for internest variation in incubation period by analysing hatching time (mean $\pm$ 1 s.e., $n = 6$ eggs for each bar) of eggs 2 (open bars), 3 (hatched bars) and 4 (filled bars) in the laying sequence relative to that of egg 1 which was treated with sesame oil plus androgens or sesame oil alone as a control. Repeated-measures ANCOVA, with volume of egg 1 as a covariate (see § 3(b)), indicated that androgen-treated eggs took longer to hatch than control eggs ($F_{1,9} = 6.50$ and $p = 0.031$). Eggs 1 and 2 in each control nest hatched simultaneously, resulting in a mean and s.e. of 0. We only analysed nests in which all of the first four eggs in the laying sequence hatched.

know whether deaths of the nestlings in these nests caused or were as a result of nest abandonment.

To control for potential treatment-independent internest variation in incubation period, we assessed time to hatching of eggs 2–4 relative to the hatching of egg 1. That is, we determined the time which elapsed between hatching of egg 1 and hatching of subsequently laid eggs in each nest (figure 1). Eggs 2–4 hatched before egg 1 in androgen-treated nests, despite the fact that egg 1 was laid on average two, four and six days before eggs 2, 3 and 4, respectively. In control nests, egg 1 hatched simultaneously with egg 2 but earlier than eggs 3 and 4. Thus, androgen treatment delayed hatching. We included volume of egg 1 as a cofactor in the statistical analysis because it correlated with the time that elapsed between hatching of eggs 1 and 2 ($F_{1,15} = 5.03$, $p = 0.040$ and $r = 0.50$).

Neither body mass nor body size (relative to age) differed significantly between control and androgen-treated nestlings at ages zero and five days (figure 2). By ten days old, however, androgen-treated nestlings were lighter and smaller than controls and remained so through to at least 20

Figure 2. Effect of yolk androgens on growth of nestling American kestrels. (a) Body mass and (b) size relative to age as defined in the text (mean $\pm$ s.e.). Repeated-measures ANOVA indicated that nestlings hatching from eggs whose yolks were treated with sesame oil plus androgens (open symbols) were lighter ($F_{1,17} = 11.14$ and $p = 0.004$) and smaller ($F_{1,17} = 9.92$ and $p = 0.006$) than nestlings hatching from eggs whose yolks were treated with sesame oil alone as a control (filled symbols). Sample sizes correspond to the number of nestlings alive at each age which we show in figure 3a. Asterisks indicate where differences between treatments were significant ($p < 0.05$) based on post hoc linear contrasts. Body mass standard error bars are smaller than the symbols at ages zero and five days.

days. The mean body mass of 15- (66.7 g) and 20-day-old (88.1 g) androgen-treated nestlings was approximately that of ten- (62.7 g) and 15-day-old (88.0 g) controls, respectively. Thus, yolk androgen treatment slowed nestling growth and, with respect to mass, growth of androgen-treated nestlings lagged five days behind controls. We provide no data on mass or size beyond age 20 days because only one androgen-treated nestling lived to age 25 days (see below).

Figure 3. Mortality of nestling American kestrels hatching from (a) egg 1 (treated), (b) egg 2, (c) egg 3 and (d) egg 4 in the laying sequence of clutches in which sesame oil as a control (filled bars) or sesame oil plus androgens (open bars) was injected into the yolk of egg 1 only. Analysis of nestling survival through to age 25 days indicated that nestlings hatching from eggs whose yolks were treated with sesame oil lived longer than nestlings hatching from eggs whose yolks were treated with sesame oil plus androgens (Breslow–Gehan–Wilcoxon, $\chi^2 = 8.00$, d.f. = 1 and $p = 0.005$). Treatment of egg 1 had no effect on mortality of nestlings hatching from subsequently laid eggs (egg 2, $\chi^2 = 1.06$, d.f. = 1 and $p > 0.2$; egg 3, $\chi^2 = 1.24$, d.f. = 1 and $p > 0.2$; and egg 4, $\chi^2 = 0.18$, d.f. = 1 and $p > 0.2$).

Using survival analysis, we analysed nestling survival rates through to age 25 days in order to determine whether yolk androgen treatment affected nestling mortality. Although nestlings from both treatments died, nestlings hatching from eggs whose yolks were treated with sesame oil lived longer than nestlings hatching from eggs whose yolks were treated with oil plus androgens (figure 3a). Thus, yolk androgen treatment increased nestling mortality. Androgen treatment of egg 1 did not affect survival rates of nestlings from eggs 2–4 (figure 3b–d).

4. DISCUSSION

Female American kestrels deposited higher concentrations of androgens into later- than first-laid eggs in the laying sequence of a clutch. Androgen treatment of first-laid eggs delayed hatching and reduced nestling growth and survival rates compared to controls. The pattern of androgen concentrations according to laying sequence was similar to that in the canary (Schwabl 1993) and red-winged blackbird (*Agelaius phoeniceus*) (Lipar *et al.* 1999) but contrasted with that in cattle egrets (*Bubulcus ibis*) (Schwabl *et al.* 1997) and zebra finches (*Taeniopygia guttata*) (Gil *et al.* 1999). However, the effects of yolk androgens were in contrast to those observed in the canary (Schwabl 1996a). Moreover, the means of body size and mass of androgen-treated nestlings (figure 2) may have been overestimated because, when nestlings become too small, they probably die and are therefore excluded from analyses. Thus, differences between treatment groups in both nestling mass and size may have been greater than those portrayed in our results (figure 2).

We do not know the mechanisms by which the injected androgens exerted their observed effects. It is possible that injections raised androgen concentrations above those that embryos are likely to experience naturally or that the injected androgens did not diffuse throughout the yolk and that embryos were exposed to a toxic ‘spike’ of exogenous androgens. The facts that doses plus endogenous concentrations in egg 1 mimicked endogenous concentrations in late-laid eggs and that hatching success, nestling body mass and size through to five days of age and mortality through to ten days of age did not significantly differ with treatment render acute toxicity of the treatment unlikely. Because testosterone injections nearly identical in procedure to those in the present study had beneficial effects on nestling canaries (Schwabl 1996a), it seems unlikely that the inhibitory effects we observed were caused simply by a lack of androgen diffusion in the yolk.

The much higher concentrations of maternal A₄ than T found in yolks raise the possibility that A₄ may be largely responsible for the observed effects. Specifically, pre-hatching exposure to A₄ may retard cell (Dąbowski & Wilmańska 1998) and embryonic (McGivern *et al.* 1996) growth or retard the hatching process itself, possibly through interactions between A₄ and the hypothalamic–pituitary–adrenal axis, as may occur in some foetal mammals (McGivern *et al.* 1996; Saoud & Wood 1997). Delayed nestling growth and higher mortality may then simply be consequences of delayed hatching. Alternatively, yolk androgens may impair nestling growth and survival independently of their effects on hatching time.

Yolk T enhances nestling begging vigour in canaries (Schwabl 1996a) and, therefore, presumably the nestlings’

ability to compete with nest-mates for food, resulting in higher growth rates. We did not quantify begging vigour in kestrels. However, if yolk androgens affect the ability to compete (by either enhancing or inhibiting it), one might expect that survival rates of untreated nestlings 2–4 would have differed between androgen-treated and control broods. This was not the case (figure 3*b–d*). Thus, at present, there is no support for the possibility that yolk androgens directly influence the competitive ability of American kestrel nestlings, although a larger sample size may reveal such an influence. Rather, androgen treatment may have reduced survival rates independently of an influence on ability to compete.

Because yolk androgen treatment delayed hatching and because late-laid eggs in the laying sequence have higher concentrations of androgens, an increasing androgen allocation with respect to laying order may increase hatching asynchrony and, subsequently, depress nestling growth and survival rates. Our results are in contrast to the situation in the canary, in which higher yolk T levels seem to mitigate the consequences of asynchronous hatching (Schwabl 1996*a*), and they challenge recent studies that assume maternal androgens necessarily benefit offspring (Schwabl *et al.* 1997; Gil *et al.* 1999). In an adaptive context, it is possible that female kestrels, which deposit high concentrations of androgens into the yolks of late-laid eggs, handicap nestlings hatching from those eggs and, therefore, reduce the energetic costs of brood reduction (see § 1). Such a tactic would be similar to asynchronous incubation of eggs and its subsequent effects on delayed hatching of late-laid eggs (Mead & Morton 1985; Clotfelter & Yasukawa 1999), the sibling developmental and competitive hierarchy (Mead & Morton 1985; Evans 1996; Nilsson & Svensson 1996; Stoleson & Beissinger 1997) and brood reduction (Lack 1947; Magrath 1990). In fact, yolk androgens may contribute to hatching patterns previously unexplained by incubation patterns alone (Bortolotti & Wiebe 1993).

However, the maternal deposition of yolk androgens may not be adaptive but rather a necessary by-product of endocrine processes during egg production, as has been argued for the production of hatching asynchrony in some species (Mead & Morton 1985). Indeed, some of our observations are not consistent with the proposition that yolk androgens are used by females to reduce the energetic costs of brood reduction. Specifically, concentrations of yolk androgens are statistically indistinguishable among eggs 2–4. This suggests that these offspring are equally handicapped, despite the fact that avian brood reduction usually results in the death of one or sometimes two nestlings (Magrath 1990). If androgens reduce the energetic costs of brood reduction, why would they not be low in all eggs except those most likely not to survive through to fledging (i.e. egg 4 or possibly eggs 3 and 4)? Other factors which vary among eggs 2–4 may contribute to a disparity in survival rates among nestlings from eggs 2–4. That is, variable allotment of yolk androgens may not be the only mechanism by which female kestrels may favour some offspring over others but rather one of several potential mechanisms operating simultaneously. For example, asynchronous onset of incubation is typical in kestrels (Beukeboom *et al.* 1988; Bortolotti & Wiebe 1993; K. W. Sockman and H. Schwabl, unpublished data)

and may be a mechanism for favouritism (Bortolotti & Wiebe 1993; Wiebe & Bortolotti 1994). In addition, because egg size affects nestling size and growth in some species (Williams 1994), variation in egg size according to laying order (Wiebe & Bortolotti 1996) may serve as a mechanism for favouring some offspring over others. Whereas differential androgen allocation may discriminate only between egg 1 and subsequently laid eggs, asynchronous incubation and variation in egg size may then interact to discriminate among eggs 2–4 and influence survival rates (as in figure 3*b–d*) and the energetic costs of brood reduction.

It is also possible that the detrimental effects of yolk androgens are greater for egg 1 than for subsequently laid eggs. This might be true if egg 1 differed from subsequently laid eggs, for example in terms of size, nutrient content, sex or some other factor that might render it particularly vulnerable to yolk androgens. If this were true and if some degree of yolk androgen deposition were necessary (i.e. for egg production), females may shelter egg 1 and shunt androgens down the hierarchy of follicles, which themselves may be less vulnerable. Egg size sometimes varies with laying order in kestrels (Wiebe & Bortolotti 1996) and other species (Henriksen 1995; Viñuela 1997). We are not aware whether laying order can affect other egg attributes, but this possibility merits further investigation and might explain why androgen concentrations in egg 1 are naturally lower than those in subsequently laid eggs.

Additional studies are necessary in order to determine the adaptive significance of yolk androgen deposition. If maternal androgen allocation is a mechanism by which females enhance their reproductive success, then androgen levels in late-laid eggs may be condition dependent and subject to facultative modification according to resource availability and other factors such as clutch size. We could not determine whether maternal androgen concentrations varied according to clutch size because we were unable to determine clutch size in most of the nests sampled (1997 and 1998 seasons). However, because clutch size sometimes declines seasonally in kestrels (Cavé 1968; K. W. Sockman and H. Schwabl, unpublished data) and because we collected yolk samples regularly over the course of the two breeding seasons, our sampling probably subsumed the natural variation in clutch size. Therefore, androgen concentrations identified in this study (table 1) probably reflect averages across clutch sizes. Future studies should identify whether androgen concentrations systematically differ with clutch size and whether such differences affect the probability of brood reduction. Further, because we do not know yolk androgen concentrations in eggs 5 and 6 of five- and six-egg clutches, the suggestions that concentrations necessarily increase with laying order and that differential yolk androgen allocation reduces the energetic costs of brood reduction may be premature. The data on maternally derived yolk androgens presented here are still useful, however, because they suggest that differential allocation within the clutch does occur and they provide a rationale for our experimental manipulation of yolk androgen levels in egg 1.

Variation in androgen allocation patterns, variation in beneficial versus deleterious actions of androgens and the

potential for condition-dependent modulation of yolk androgen contents underscore the possibility that the way in which maternal hormones regulate offspring survival or in which female endocrine processes secondarily affect offspring depends on reproductive strategy (Schwabl *et al.* 1997) and ecological circumstance. Such transgenerational effects of steroid hormones (Bern 1990) may have helped to evolutionarily shape the endocrine regulation of reproduction (Schwabl *et al.* 1997).

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