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Intraclutch Variation in Testosterone Content of Red-winged Blackbird Eggs

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Hatching asynchrony occurs in many avian species, often because parents initiate incubation before the last egg in the clutch has been laid. Because parents typically begin to feed individual young as soon as they hatch, earlier-hatched young start to grow before their younger siblings have hatched. This frequently results in a size hierarchy that is hatching-order dependent among nestlings (Bryant 1978, Richter 1984, Greig-Smith 1985, Stokland and Amundsen 1988). Because access to food brought by parents is largely dependent on the size-related competitive abilities of the young (Ryden and Bengtsson 1980, Smith and Montgomerie 1991, McRae et al. 1993, Malacarne et al. 1994, Kacelnik et al. 1995, Price and Ydenberg 1995), the youngest siblings in a clutch often are at a significant disadvantage.

Much debate has centered on whether hatching asynchrony is adaptive, and a number of hypotheses to support its adaptive significance have been proposed (Magrath 1990, Stoleson and Beissinger 1995). Many of these hypotheses are based on the idea that asynchrony promotes the survival of "core" offspring that have hatched earlier than their siblings. This argument, which depends on the fact that the last-hatched young is often the one that dies, supposes that the benefits of hatching asynchrony derive directly from its role in producing a size hierarchy among nestlings. According to Mock and Forbes (1995), this overproduction of brood members may

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be adaptive in that it provides a means by which parents can: (1) take advantage of unusually abundant resources by raising an extra (last-hatched) offspring, and/or (2) provide replacements if any core offspring fails to survive or happens to develop poorly.

Others have proposed that the adaptive significance of hatching asynchrony is not necessarily related to the production of a size hierarchy among nestlings, or that asynchrony is a nonadaptive consequence of underlying physiological or behavioral mechanisms (Magrath 1990, Stoleson and Beissinger 1995). If either of these cases is true, then one might expect to find mechanisms that have evolved to mitigate the detrimental effects of hatching asynchrony and enhance the survival of last-hatched offspring. If, on the other hand, the adaptive significance of hatching asynchrony is indeed related to the production of a size hierarchy, then mechanisms that mitigate the effects of that hierarchy may at first seem counterproductive. However, the existence of two such apparently incongruous mechanisms, i.e. the production of a size hierarchy and the mitigation of its effects, may in fact give parents the means to adjust the probability of survival for any given nestling more precisely than does hatching asynchrony alone. For example, Howe (1976) found that Common Grackles (*Quiscalus quiscula*) exhibit hatching asynchrony and nestling starvation, but that they also produce larger eggs near the end of the laying order. He suggested that although these traits appear to be antagonistic, they actually work together to maximize reproductive success. According to Howe, the production of larger eggs near the end of the laying sequence allows parents finer control over their own reproductive success by keeping the youngest offspring alive for a longer period of time, thereby extending the amount of time available to determine whether an entire brood can be raised.

Another mechanism that could work in opposition to size hierarchies induced by hatching asynchrony is differential apportionment of steroid hormones to egg yolks. Yolk testosterone concentrations increase with laying order in Common Canaries (*Serinus canaria*), a species in which partial hatching asynchrony produces a size hierarchy among nestlings (Schwabl 1993). Interestingly, the social rank of captive juvenile canaries varies with laying order and covaries positively with naturally varying yolk testosterone concentrations (Schwabl 1993). In addition, exogenous testosterone increases nestling growth rates, and chicks from eggs with high maternal concentrations of testosterone grow faster than those from eggs with low concentrations of the hormone (Schwabl 1996). These results suggest that female Common Canaries vary the testosterone concentration of their eggs in a way that mitigates the effects of hatching asynchrony on the competitive abilities of last-hatched offspring (Schwabl 1993). Similarly, allocation of less testosterone to last-laid eggs (which usually produce last-hatched young) could reinforce the effects of hatching asynchrony by further enhancing the probability of survival of early hatched young. In fact, testosterone concentration decreases with laying order in the Cattle Egret (*Bubulcus ibis*; Schwabl et al. 1997), a species in which the last-hatched young often is subjected to high levels of sibling aggression, sometimes with fatal results (Ploger and Mock 1986).

In this paper, we present data suggesting that a hormonal mechanism to mitigate the detrimental effects of hatching asynchrony on last-hatched young is also present in the Red-winged Blackbird (*Agelaius phoeniceus*). This species exhibits partial hatching asynchrony such that the typical three- to five-egg clutch hatches over a period of two to three days (Hengeveld 1989, Yasukawa and Searcy 1995). The resulting size hierarchy of nestlings frequently leads to mortality from starvation (Blank and Nolan 1983, Hengeveld 1989, Patterson 1991), and when starvation does occur, it primarily affects the young produced from last-laid eggs (Blank and Nolan 1983, Hengeveld 1989). Interestingly, nestling starvation rates in Red-winged Blackbirds vary according to maternal age. In broods of yearling females, 57% of nestlings that hatched from last-laid or penultimate eggs starved, compared with 13% for older females (Blank and Nolan 1983). This variation coincides with the fact that older females, but not yearlings, produce significantly larger eggs near the end of the laying order (Blank and Nolan 1983), suggesting that egg mass, and therefore nestling mass (Williams 1994), influences nestling survival.

Because the hatching pattern in Red-winged Blackbirds is similar to that in Common Canaries, we predicted that yolk testosterone deposition within clutches would increase with laying order, as it does in canaries. Such a pattern would ensure that the nestling that is most disadvantaged by hatching asynchrony would receive the highest dose of yolk testosterone. Furthermore, because intraclutch patterns of egg mass vary among female Red-winged Blackbirds, we predicted that females would also vary in the amount of testosterone they deposit into their eggs.

Methods.—We collected 14 complete three-egg clutches of Red-winged Blackbirds from nests at four different sites near Bloomington, Indiana (ca. 39°10′N, 86°30′W) between 1 May and 27 June 1995. Because epaulets of female Red-winged Blackbirds increase in brightness with age (Johnsen et al. 1996), we recorded maternal age by comparing epaulet brightness with a series of 12 ranked photographs of increasingly bright epaulets and found that all of the females from which eggs were collected were in at least their second breeding season. We found nests while they were being constructed and visited them daily, recording laying order by numbering each egg on the day it was laid. Clutches were collected one day after the final egg had been laid, thereby minimizing embryonic development. Eggs were frozen whole at −20°C and stored until December 1995, when analysis of testosterone concentration was performed.

We separated the yolk of each egg by taking advantage of the fact that albumin thaws more quickly than yolk. After recording the mass of each yolk, we homogenized it by swirling with a mini-spatula to prepare it for analysis of testosterone concentration, which we performed with the competitive-binding radioimmunoassay method outlined by Wingfield and Farner (1975). The details of this procedure are summarized below. First, 2,000 cpm of ^{3}H-testosterone (New England Nuclear) were added to 10-mg samples of yolk to serve as an internal reference for the detection of recovery percentages following extraction and chromatography. The endogenous and tritiated steroids were then extracted with petroleum and diethyl ethers, followed by a rinse with 95% ethanol to remove excess lipids (Schwabl 1993). To separate the testosterone from other endogenous steroids, the extracts were evaporated with nitrogen gas and redissolved in 10% ethyl acetate in isooctane, then applied to chromatography columns consisting of a celite : ethylene glycol:propylene glycol upper phase and a celite : water lower phase. Following the elution of 5α-dihydrotestosterone with 10% ethyl acetate in isooctane, the testosterone fraction was eluted with 20% ethyl acetate in isooctane, evaporated with nitrogen gas, and redissolved in a phosphate buffer. The concentration of testosterone was measured by radioimmunoassay with ^{3}H-testosterone standard and an antibody specific to testosterone and 5α-dihydrotestosterone (Wien Laboratories). Duplicate values of each sample were compared to a standard curve that ranged in concentration from 500 to 1.95 pg. The analysis of a standard sample containing a known amount of testosterone yielded a value that was within 1% of its expected value. Recovery values ranged from 38 to 82% and averaged 73%.

We used repeated-measures ANOVA to determine if differences in hormone concentration and whole-yolk steroid content existed among successive eggs in a clutch. Multiple contrasts between successive eggs in the laying order also were made. We used one-way ANOVA to determine whether females differed in the average amount of testosterone they allocated to their eggs.

Results.—As predicted, testosterone concentrations in yolk increased with laying order within the clutch. Individual eggs contained testosterone at levels that ranged from 1.61 to 125.98 pg/mg of yolk, and concentrations differed significantly with laying order (F = 17.12, df = 2 and 26, $P < 0.001$; Fig. 1). Multiple contrasts of mean testosterone concentration between positions in the laying sequence revealed significant differences between the first and third ($P < 0.001$) and the second and third ($P < 0.001$) eggs. Whole-yolk amounts of testosterone, calculated by multiplying testosterone concentration by the mass of the whole yolk, averaged 26.6, 37.2, and 47.0 ng/yolk, respectively, for the first through third eggs in the laying order. Significant differences across the laying order were found (F = 8.15, df = 2 and 12, P = 0.006; statistical analysis of this variable was limited to a subset of our sample because several yolks could not be weighed accurately owing to damage incurred during handling).

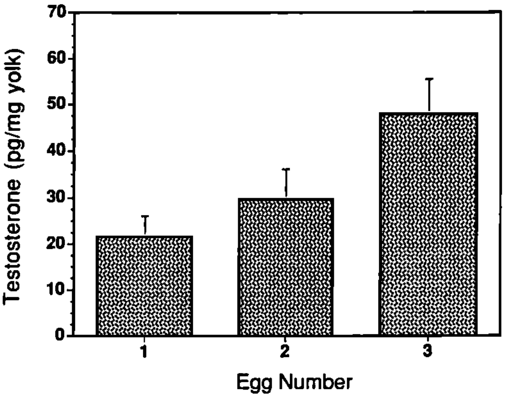

FIG. 1. Mean testosterone concentration (whiskers = 1 SE) versus position in the laying order (egg number) of Red-winged Blackbird eggs. The average concentrations in the first through third eggs were 22.5, 30.3, and 50.5 pg/mg of yolk, respectively; n = 14 for each egg number.

Females varied in the amount of testosterone they allocated to their eggs, as measured by the average concentration of testosterone across all three eggs in the clutch (F = 4.38, df = 13 and 28, $P < 0.001$). A plot of testosterone concentration versus position in the laying order for individual females (Fig. 2) suggests that females also vary in their intraclutch patterns of testosterone deposition, i.e. the difference in testosterone concentrations between the first and last eggs of the clutch. Testosterone levels increased between the first and last eggs in 13 out of 14 clutches, indicating that the pooled data are representative of individual clutches.

Discussion.—Our results show that testosterone concentrations in yolk increase with laying order in clutches of Red-winged Blackbirds, a species in which hatching asynchrony often leads to a size hierarchy among nestlings. As an example of this asynchrony, one study reported that the interval between the hatching of the first and last eggs in a clutch ranged from 0 to 48 h, with a mean of 17.9 ± SE of 1.2 h (Blank and Nolan 1983). In a separate study of members of this same population, the difference in mass between the first- and last-hatched chicks at the

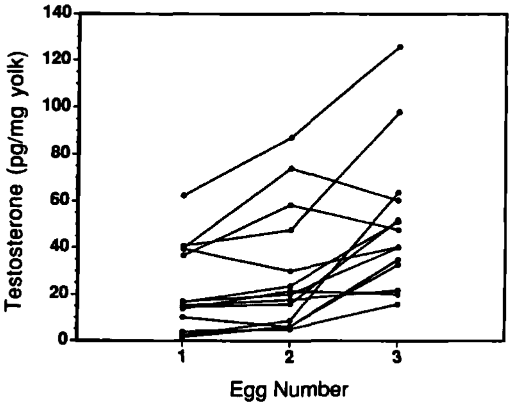

FIG. 2. Testosterone concentration of Red-winged Blackbird eggs versus position in the laying order for clutches of individual females.

time the last egg hatched was 1.67 g in broods of three and 2.16 g in broods of four (Hengeveld 1989). The mass of Red-winged Blackbird nestlings immediately after hatching is approximately 3 g (pers. obs.), so the differences mentioned above represent a substantial proportion of the total mass of the last-hatched young. Based on these mass differences, the ability of last-hatched nestlings to obtain food from their parents in competition with their siblings probably is reduced, which may be responsible for the starvation of last-hatched young (i.e. brood reduction) that often occurs in this species (Robertson 1972, Blank and Nolan 1983, Hengeveld 1989, Patterson 1991).

As discussed earlier, the existence of adaptations capable of mitigating the effects of asynchronous hatching on the survival of the youngest and smallest nestling in a clutch should not be surprising. Given that larger eggs produce larger nestlings (Williams 1994) that presumably have greater competitive abilities, the increase in egg mass with laying order in Red-winged Blackbirds (Blank and Nolan 1983, Weatherhead 1985, Hengeveld 1989) may represent such an adaptation. Work must now be done to establish whether a relationship exists between yolk testosterone and the competitive abilities and survival rates of nestlings. If a positive correlation is found, then the apportionment of greater quantities of testosterone to last-laid eggs (see Fig. 1) may also be a mechanism capable of moderating the disadvantages of the last-hatched nestling.

Recently, Forbes et al. (1997) presented evidence that last-laid eggs in Red-winged Blackbird clutches provide insurance in case an earlier-laid egg fails to hatch. In broods without hatching failure, the mortality rate for last-hatched offspring was more than five times higher than that of their siblings. Mortality was significantly reduced for last-hatched offspring, however, if an egg laid earlier in the clutch did not hatch. If the production of an "extra" nestling has evolved as insurance for the replacement of a sibling that fails to survive, then it is possible that the higher level of testosterone in the egg that produces the "insurance" offspring contributes to that offspring's ability to compete for food once it has hatched.

The presence of traits that tend to work in opposition to the effects of a size hierarchy among nestlings does not help to explain whether hatching asynchrony is adaptive or nonadaptive. As noted earlier, such traits may be expected in either case. To determine what the adaptive significance (if any) of hatching asynchrony may be, simultaneous investigations of all known traits associated with laying order, hatching order, and nestling quality should be undertaken, including the possible effects of yolk testosterone on nestling survival. These effects, which may include alterations of begging behavior, muscle development, and growth rates, remain unexplored in wild avian species. Additionally, variation among females in the pattern of intraclutch testosterone deposition and the effects of this variation on parental fitness should be investigated. An experimental manipulation in which the normal pattern of testosterone deposition is altered would be a first step in asking whether and how deviations from the normal pattern affect these variables.

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