THE ROYAL SOCIETY

Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird *Agelaius phoeniceus*

Joseph L. Lipar[*] and Ellen D. Ketterson

Department of Biology and Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN 47405, USA

Hatching asynchrony in avian species often leads to the formation of a size hierarchy that places last-hatched nestlings at a significant disadvantage. The hatching muscle (musculus complexus) is responsible for breaking the shell during hatching and for dorsal flexion of the neck during begging. An increase in its strength in last-hatched nestlings could mitigate the effects of hatching asynchrony by reducing the time required for hatching or enhancing the effectiveness of begging for parentally delivered food or both. We have previously found that yolk testosterone concentration increases with laying order in the red-winged blackbird *Agelaius phoeniceus*. In this study, we investigated the hypothesis that yolk testosterone has anabolic effects on the development of the complexus, thereby influencing competition among asynchronously hatched nestlings. We found that both yolk testosterone concentration and relative complexus mass (complexus mass/nestling body mass) increased with laying order and that these two variables were positively correlated in both newly hatched nestlings and in two-day-old broods. Moreover, direct injections of testosterone into egg yolks resulted in an increase in relative complexus mass, while injections of flutamide, a testosterone antagonist, resulted in a decrease in relative complexus mass. Neither yolk testosterone concentration nor relative complexus mass differed between male and female nestlings.

Keywords: musculus complexus; testosterone; hatching asynchrony

1. INTRODUCTION

Many avian species exhibit asynchrony in the hatching of their eggs, often because parents initiate incubation before the last egg in the clutch has been laid. Because parents typically begin to feed nestlings as soon as they hatch, earlier hatched young start to grow before their later laid siblings have hatched. This frequently results in a hatching order-dependent size hierarchy among nestlings (Bryant 1978; Richter 1984; Greig-Smith 1985). Because access to food brought by parents is largely dependent on the size-related competitive abilities of the young (Ryden & Bengtsson 1980; Kacelnik *et al.* 1995; Price & Ydenberg 1995), the youngest siblings in a brood are often at a significant disadvantage.

Red-winged blackbirds exhibit hatching asynchrony such that clutches of three to five eggs hatch over a period of one to three days (Yasukawa & Searcy 1995). This asynchrony can result in a 40% or more difference in mass between the first- and last-hatched chicks at the time the last egg hatches (Hengeveld 1989). It is therefore likely that the ability of last-hatched nestlings in obtaining parentally delivered food in competition with their siblings is reduced. Forbes *et al.* (1997) found that the mortality of last-hatched offspring was significantly greater in clutches in which all eggs hatched than it was in clutches in which at least one egg did not hatch. This suggests that last-hatched nestlings of this species may provide insurance to parents in case earlier laid eggs fail to hatch. If the production of an 'extra' nestling has

evolved as insurance for replacing older siblings that fail to survive, then it is possible that females manipulate the development of that nestling in order to enable it to compete better with its siblings for parentally delivered food when the occasion arises.

The musculus complexus, a large, dorsally located neck muscle that overlies the spinalis and biventer cervicus, serves two functions in avian development. During hatching it provides the force necessary for breaking the shell through dorsal and lateral head movements (Gross 1985). After hatching, the complexus allows dorsal flexion and extension of the neck during begging (Ashmore *et al.* 1973). Because the complexus controls the movements involved in both hatching and begging, an increase in its strength may translate into either a shortened duration of hatching or an increased ability for obtaining parentally delivered food.

Steroid hormones, particularly testosterone, influence the development of the vertebrate musculoskeletal system (Kawata 1995). For example, the administration of testosterone increases overall muscle mass in the forelimb musculature of the leopard frog *Rana pipiens* (Blackburn *et al.* 1995; Sidor & Blackburn 1998) and the sonic muscle in two species of teleost fishes, *Porichthys notatus* (Brantley *et al.* 1993) and *Cynoscion regalis* (Connaughton & Taylor 1995). Recent investigations have shown that a variety of steroid hormones, including testosterone, are present in eggs of the red-winged blackbird *Agelaius phoeniceus* (Lipar *et al.* 1999a) and the canary *Serinus canaria* (Schwabl 1993). Interestingly, the concentration of yolk testosterone increases with laying order in both of these species, each of which exhibits some degree of hatching asynchrony.

If we assume that there are selective advantages in the incubation patterns responsible for asynchronous hatching

[*]Author and address for correspondence: School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA (jlipar@wsu.edu).

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in the red-winged blackbird (Stoleson & Beissinger 1995), then we might expect to find that mechanisms have evolved for alleviating the detrimental effects of hatching asynchrony and enhancing the probability of survival for last-hatched offspring. Indeed, yolk testosterone concentration is positively correlated with social rank in juvenile canaries (Schwabl 1993), and the addition of exogenous testosterone to eggs of this species increases nestling growth rates (Schwabl 1996). Similarly, an increase in the strength of the musculus complexus during either hatching or begging may confer competitive and, therefore, fitness advantages to selected nestlings. We therefore investigated the hypothesis that yolk testosterone has anabolic effects upon the development of the complexus. In order to examine the correlative relationship between yolk testosterone concentration and the mass of the complexus, we took yolk samples from viable eggs and allowed the eggs to hatch before we collected the complexus from both newly hatched nestlings and two-day-old broods. We predicted that (i) both complexus mass and yolk testosterone concentration would increase with laying order, and (ii) yolk testosterone concentration would be positively correlated with the mass of the complexus at both ages. In addition, we manipulated the amount of testosterone available to the developing embryo via direct injections of either testosterone or fluta-mide, a testosterone antagonist, into the yolks of newly hatched eggs. We predicted that the injection of testosterone would result in an increase in complexus mass, while the injection of flutamide would result in a decrease in complexus mass.

2. METHODS

(a) *General methods*

Correlative data were collected from 19 four-egg clutches of red-winged blackbirds, ten clutches in 1997 and nine clutches in 1999. Experimental manipulations were performed on 22 additional clutches in 1999. All nests were located near the Cedar Point Biological Station of the University of Nebraska (41° 20′ N, 101° 43′ W) and were examined between 18 May–4 July 1997 and 18 May–20 June 1999. We found nests while they were being built, visited them daily during laying and marked each egg in order to indicate laying order. We related individual nestlings to the eggs from which they hatched by observing the hatching of each egg.

(b) *Yolk sample collection*

Yolk samples were taken from eggs 1 and 2 on the day that egg 2 was laid and from eggs 3 and 4 on the day that egg 4 was laid. Red-winged blackbirds typically do not initiate incubation until after the laying of the penultimate egg (Yasukawa & Searcy 1995); therefore, no egg would have been subjected to incubation for longer than 24 h when biopsied. (See Lipar *et al.* (1999b) for justification that steroids present in the yolk are not of embryonic origin.) Yolk samples (10 μl) were collected by placing eggs over a fibre-optic light source and penetrating the shell and yolk membranes with a sterile butterfly infusion set (25 3/4 in length, 12 in tubing (1 in = 0.0254 m), Abbott Laboratories, North Chicago, IL, USA) attached to a 1 ml tuberculin syringe (Schwabl 1993). Because steroid levels vary between layers of individual yolks, care was taken to draw samples from the same layer in each yolk (Lipar *et al.* 1999b). Eggshells were patched with a small

square of Opsite breathable wound dressing (Briggs Corporation, Des Moines, IA, USA). Individual samples were homogenized in 1.5 ml microcentrifuge tubes with 500 μl of water. Homogenization was facilitated by the addition of several glass beads.

(c) *Experimental manipulation of yolk testosterone*

The first- and last-laid eggs of four-egg clutches were manipulated according to one of three treatments. In control clutches, both eggs were controls and received an injection of vehicle (5 μl of 70% ethanol). In testosterone-treated clutches, first-laid eggs were injected with testosterone (110 ng) in vehicle in order to approximate the highest levels of yolk testosterone concentration in this species (Lipar *et al.* 1999a) and last-laid eggs were injected controls. In flutamide-treated clutches, first-laid eggs were injected controls and last-laid eggs were injected with flutamide, an anti-androgenic compound that blocks androgen receptor activation, in vehicle. The dose used (0.08 μmol) has anti-androgenic effects on the development of the domestic fowl (Burke 1996). Injections were made on the day each egg was laid using the same procedure as described above for the collection of yolk samples.

(d) *Radioimmunoassay*

All samples were analysed for the presence of testosterone with a competitive-binding radioimmunoassay (Wingfield & Farner 1975; Schwabl 1993). A separate assay was performed in each year of study. Approximately 2000 cpm (counts per minute) ³H-testosterone (New England Nuclear, Boston, MA, USA) was added to each sample for the calculation of recoveries. Steroids were extracted with petroleum and diethyl ethers (30:70%), followed by precipitation with 95% ethanol in order to remove excess lipids. The extracts were applied to chromatography columns that consisted of a celite:ethylene glycol:propylene glycol upper phase and a celite:water lower phase. The testosterone fraction was eluted with 20% ethyl acetate in iso-octane. Hormone concentration was measured by competitive-binding radioimmunoassay with tritiated hormone and a specific antibody (Wien Laboratories, Succasunna, NJ, USA). Duplicate values of each sample were compared with a standard curve that ranged from 1.95–500 pg in concentration. The recovery values averaged 63% for the 1997 samples and 48% for the 1999 samples. The intra-assay variation, which was calculated as the coefficient of variation of values obtained from standard samples of known concentration, was 11% for the 1997 samples and 12% for the 1999 samples. The inter-assay variation was 16%.

(e) *Muscle collection*

Nests were visited approximately five times daily in 1997 and nestlings were assigned to their appropriate position in the laying order and marked accordingly. The entire brood was collected *ca.* 48 h after hatching of the first nestling. Because of their asynchronous hatching, the ages of nestlings at the time of collection varied within a brood. Body masses were recorded to the nearest 0.05 g. Nestlings were sacrificed (inhalation of Meto-fane) and placed in a 10% buffered formalin solution (Sigma, St Louis, MO, USA) for storage until dissection. After excision, muscles were patted dry and masses were recorded to an accuracy of 0.0001 g. Relative complexus mass was recorded as the mass of the complexus divided by body mass. In addition to the complexus, the iliotibialis, which is responsible for flexion of the hip and extension of the knee and lower leg and the gizzard (minus its contents) were also excised, patted dry and weighed.
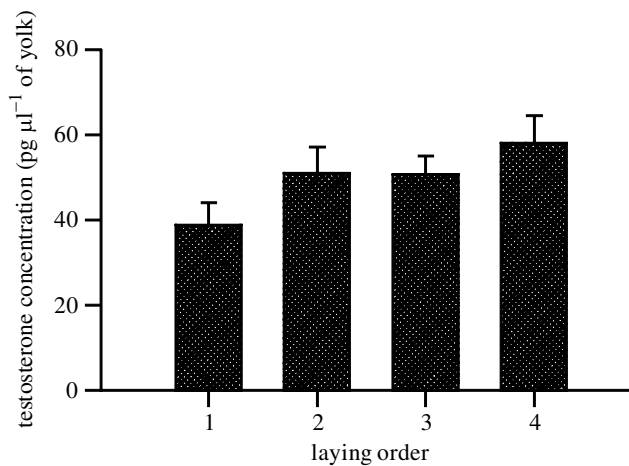
Figure 1. Mean testosterone concentration in yolk samples of red-winged blackbird eggs collected in both 1997 and 1999. Yolk testosterone concentration increased with laying order (repeated-measures ANOVA, $F_{3,75} = 5.22, p = 0.0031$ and $n = 19$). Error bars represent one standard error of the mean.

Exact hatching times were recorded for all nestlings in 1999. Individual nestlings were collected as they hatched and were transported from the field site to a station laboratory where dissection was performed. We recorded the relative complexus mass for each nestling and the time that elapsed between hatching and dissection. This time-lag ranged from 1.00–5.75 h. The sex of each nestling was also recorded based on observation of the gonads.

(f) *Statistics*

Comparisons of yolk testosterone concentration, nestling body mass, complexus mass and relative complexus mass in both correlative and experimental clutches were made across the laying order with a repeated-measures analysis of variance in both 1997 and 1999. This method was also used to analyse intra-clutch variation in relative iliotibialis mass (1997), relative gizzard mass (1997) and relative complexus mass in experimental clutches (1999). When appropriate, post-hoc comparisons were made using the Student–Newman–Keuls method.

We used analysis of variance to compare average yolk testosterone concentration and average relative complexus mass among females. Because females varied in the amount of testosterone they allocated to their eggs (1997, $F_{9,30} = 4.33$ and $p = 0.001$, and 1999, $F_{8,27} = 4.12$ and $p = 0.003$) and in the mean value of relative complexus mass in their broods (1997, $F_{9,30} = 2.20$ and $p = 0.051$, and 1999, $F_{8,27} = 2.20$ and $p = 0.061$), we adjusted the data for both variables into the deviation of each data point from the mean value of its clutch. These modified variables were used in univariate linear regressions in both 1997 and 1999.

A *t*-test was used to compare yolk testosterone concentration and relative complexus mass in male and female nestlings (1999). Because these variables did not differ between the sexes (see § 3), nestling sex was not included as a cofactor in subsequent analyses.

A univariate linear regression was used to investigate the relationship between the time-lag, which was the amount of time that elapsed between hatching and excision of the muscle, and relative complexus mass in 1999. Because relative complexus mass was negatively correlated with the time-lag ($F_{1,160} = 8.85$,
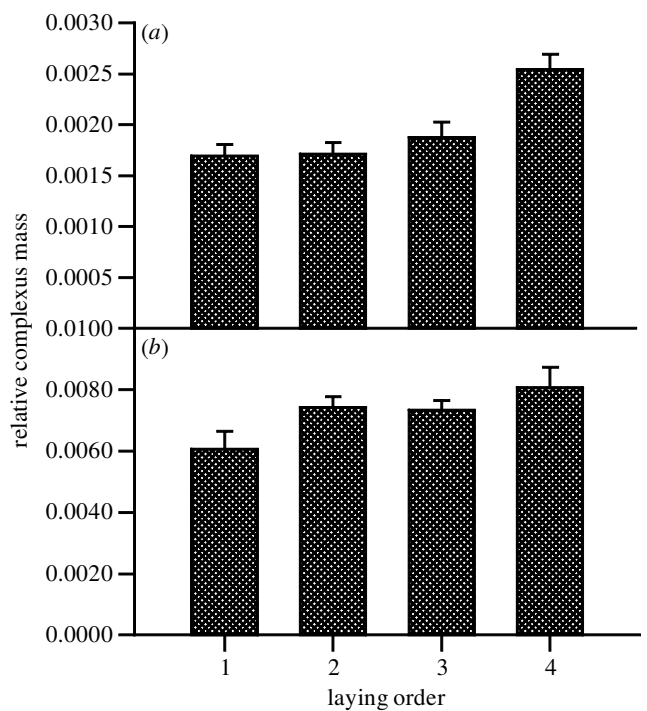

Figure 2. Relative complexus mass (complexus mass/nestling body mass) versus laying order in asynchronously hatched clutches of red-winged blackbirds (*a*) collected 48 h after hatching of the first egg in 1997 and (*b*) in newly hatched red-winged blackbird nestlings in 1999. Relative complexus mass increased with laying order (repeated-measures ANOVA) in both 1997 ($F_{3,39} = 20.4, p < 0.0001$ and $n = 10$) and 1999 ($F_{3,35} = 4.0, p = 0.0200$ and $n = 9$). The time-lag was used as a covariate in 1999. Error bars represent one standard error of the mean.

$p = 0.003$ and $r = 0.230$), time-lag was included as a covariate in the analyses of the 1999 data.

3. RESULTS

(a) *Yolk testosterone*

Yolk testosterone concentration increased with laying order in both 1997 ($F_{3,39} = 6.15$ and $p = 0.003$) and 1999 ($F_{3,35} = 4.30$ and $p = 0.015$). There was a significant year effect on yolk testosterone concentration ($F_{1,75} = 8.46$ and $p = 0.010$), but this effect disappeared when the values from the two years were standardized according to samples of known concentration ($F_{1,75} = 2.31$ and $p = 0.147$), indicating that this effect was due to inter-assay variation. Variation in yolk testosterone concentration across the laying order was maintained when the standardized data from 1997 and 1999 were combined ($F_{3,75} = 5.22$ and $p = 0.0031$) (figure 1). The testosterone concentration in the first egg was significantly lower than in each of the subsequently laid eggs ($p < 0.05$).

(b) *Hatching muscle*

Relative complexus mass (complexus mass/body mass) varied across the laying order in 1997 ($F_{3,39} = 20.4$ and $p < 0.001$) (figure 2) and was significantly greater in the last-hatched nestling than in the other nestlings ($p < 0.05$). Absolute complexus mass did not vary across the laying order ($F_{3,39} = 1.96$ and $p = 0.144$), while nestling
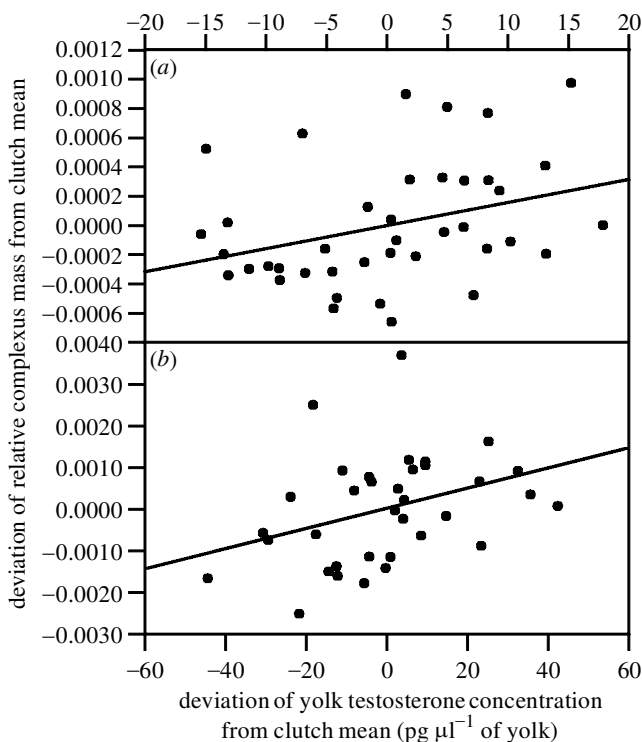
Figure 3. Deviation of relative complexus mass from the mean of its clutch versus deviation of yolk testosterone concentration from the mean of its clutch. A univariate linear regression revealed a significant positive correlation between the two variables in both (*a*) 1997 ($F_{1,39} = 4.77, p = 0.035$ and $r = 0.334$) and (*b*) 1999 ($F_{1,35} = 3.38, p = 0.046$ and $r = 0.412$). The time-lag was used as an additional independent variable in 1999.

Figure 4. Relative complexus mass (complexus mass/ nestling body mass) in the first- and last-laid eggs of control, testosterone-treated and flutamide-treated clutches. The relative complexus mass was significantly greater in the last-hatched nestling in control clutches ($F_{1,15} = 7.67, p = 0.032$ and $n = 8$). The injection of testosterone into the yolks of first-laid eggs eliminated this difference ($F_{1,17} = 1.87$, $p = 0.214$ and $n = 9$) as did the injection of flutamide into last-laid eggs ($F_{1,9} = 0.28, p = 0.632$ and $n = 5$). A repeated-measures ANOVA was used with the hatching-to-muscle excision time-lag as a covariate. Error bars represent one standard error of the mean.

body mass decreased significantly across the laying order ($F_{3,39} = 44.5$ and $p < 0.001$) due to variation in nestling age. Neither relative iliotibialis mass ($F_{7,31} = 1.79$ and $p = 0.180$) nor relative gizzard mass ($F_{7,31} = 1.52$ and $p = 0.239$) varied across positions in the laying order.

Relative complexus mass also increased significantly with laying order in 1999 ($F_{3,35} = 4.0$ and $p = 0.020$) (figure 2). Pairwise multiple comparisons revealed that the relative complexus mass in the fourth nestling was significantly greater than in the first nestling ($p < 0.05$). Absolute complexus mass also increased significantly with laying order ($F_{3,35} = 3.74$ and $p = 0.025$), while nestling body mass did not vary across the laying order ($F_{3,35} = 0.31$ and $p = 0.819$).

The deviation of relative yolk testosterone concentration from the mean of its clutch was positively correlated with the deviation of relative complexus mass from the mean of its clutch in both 1997 ($F_{1,39} = 4.77, p = 0.035$ and $r = 0.334$) (figure 3) and 1999 ($F_{1,35} = 3.38, p = 0.046$ and $r = 0.412$) (figure 3).

(c) *Nestling sex*

Yolk testosterone concentration averaged 58.4 pg μl^{-1} of yolk in eggs that produced female nestlings ($n = 16$) and 62.7 pg μl^{-1} of yolk in eggs that produced male nestlings ($n = 20$) and did not differ between the sexes ($t_{34} = 0.446$ and $p = 0.658$). Similarly, relative complexus mass averaged 0.00709 g in female nestlings ($n = 16$) and
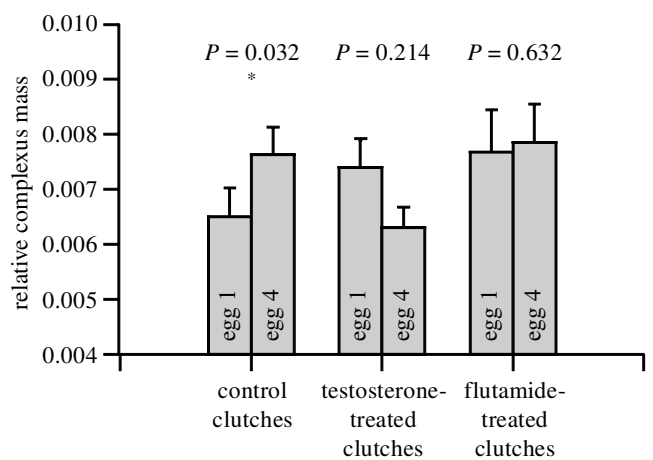
0.00730 g in male nestlings ($n = 20$) and did not differ between the sexes ($t_{34} = 0.376$ and $p = 0.709$).

(d) *Experimental manipulations*

Due to variation among females in the mean value of the relative complexus mass in their broods (see § 2(f)), our analyses were limited to within-clutch comparisons of nestlings. As expected, relative complexus mass was significantly greater in the last-hatched nestling in control clutches ($F_{1,15} = 7.67$ and $p = 0.032$) (figure 4). The injection of testosterone into the yolks of first-laid eggs eliminated this difference ($F_{1,17} = 1.87$ and $p = 0.214$) (figure 4) as did the injection of flutamide into last-laid eggs ($F_{1,9} = 0.28$ and $p = 0.632$) (figure 4). There was no difference in sex ratio between the first- and last-laid eggs for any of the treatments (Fisher's exact test, $p = 1.00, p = 1.00$ and $p = 0.286$, respectively).

4. DISCUSSION

As predicted, both yolk testosterone concentration (figure 1) and relative complexus mass (figure 2) increased with laying order and yolk testosterone concentration was positively correlated with relative complexus mass (figure 3). Moreover, the injection of testosterone into egg yolks resulted in an increase in relative complexus mass, while injections of flutamide, a testosterone antagonist, resulted in a decrease in relative complexus mass (figure 4). These results support the hypothesis that yolk testosterone has anabolic effects on the development of the complexus and suggest that female red-winged blackbirds vary the amount of testosterone in their eggs to influence the development of the complexus and mitigate the effects of hatching asynchrony on last-hatched offspring.

During hatching, the complexus provides the force necessary for breaking the shell through dorsal and lateral head movements (Gross 1985). If we assume that larger muscles are stronger, then an increase in complexus mass may allow a hatchling to break through the shell more efficiently. If the amount of time it takes later laid eggs to hatch is shortened because of an increased relative complexus mass (figure 2), then the difference in body mass that results from hatching asynchrony would be reduced. The size-related competitive disadvantage that last-hatched nestlings would otherwise have incurred would be diminished and their probability of survival would increase.

After hatching, the complexus is responsible for the dorsal flexion and extension of the neck when the nestling is fed by its parents (Ashmore *et al.* 1973). Our results show that, in broods that are *ca.* 48 h old, the youngest nestlings have a larger relative complexus mass. If larger muscles are stronger, then an increase in complexus mass may allow a nestling to lift its head more rapidly or more frequently or maintain the begging posture for a longer period. Because the competitive abilities of nestlings are largely dependent on their begging activity (Gottlander 1987; Kacelnik *et al.* 1995; Price *et al.* 1996), a larger complexus might confer a competitive advantage upon last-hatched nestlings. This would also serve to mitigate the detrimental effects that hatching asynchrony could have on last-hatched nestlings.

In the first year of the study we collected whole broods *ca.* 48 h after the hatching of the first-laid egg. As a result, the nestlings in any given brood were not all the same age. Because the mass of the complexus declines after hatching, presumably owing to the loss of fluid (Ashmore *et al.* 1973), it is likely that younger nestlings retain a larger proportion of the lymph that infiltrated the muscle at hatching. We cannot be certain from this data how much of the observed difference in relative complexus mass is attributable to variation in lean muscle mass among nestlings and how much to the residual presence of lymph. However, the increase in relative complexus mass with laying order in newly hatched nestlings in the second year of the study suggests that variation in lean muscle mass is indeed responsible for overall variation in relative complexus mass. Further support for this hypothesis comes from studies investigating the development of the complexus in the European starling *Sturnus vulgaris*, a species which exhibits a pattern of hatching asynchrony that is similar to that of the red-winged blackbird. Although relative complexus mass increases with laying order in this species, there is no variation in the ratio of dry:wet complexus mass, which is a measurement of the proportion of water in the muscle, across the laying order (J. L. Lipar, unpublished data). Regardless of whether intraclutch differences in relative complexus mass result from different rates of muscle growth due to variation in yolk testosterone concentration or from fluid loss, an increased complexus mass should help last-hatched nestlings compete with their older, more-developed siblings, provided that an increase in complexus mass translates into an increase in the strength of the muscle.

We found that relative complexus mass did not differ between male and female nestlings in this species, despite the fact that the red-winged blackbird is sexually dimorphic in adulthood. It is interesting to note that the mass of this muscle is correlated with position in the laying order but not with the sex of the offspring, despite the fact that its development is regulated by testosterone, a sex steroid that is generally involved in the development of physiological and behavioural differences between the sexes (Kawata 1995).

The data presented here indicate that an increase in testosterone in eggs that produce last-hatched nestlings results in an increase in the mass of the complexus. However, the present study did not address the functional advantage of a larger complexus. Future studies will need to determine the relationships between yolk testosterone concentration and the physiological characteristics and performance of this muscle so that a more complete understanding of its role in sibling competition and nestling survival can be attained.

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