YOLK ANDROGENS AND DEVELOPMENT

IN AMERICAN KESTREL NESTLINGS

By

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A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Raptor Biology

Boise State University

May 2009

BOISE STATE UNIVERSITY GRADUATE COLLEGE

DEFENSE COMMITTEE APPROVAL

of the thesis submitted by

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Thesis Title: Yolk androgens and development in American kestrel nestlings

Defense Date: 30 March 2009

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ACKNOWLEDGEMENTS

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CHAPTER 1: YOLK ANDROGENS AND DEVELOPMENT

IN AMERICAN KESTREL NESTLINGS

Abstract

Maternally derived yolk androgens affect many aspects of phenotypic development. To

explore this phenomenon I injected clutches of American kestrels (*Falco sparverius*) with

one of three different dosages of testosterone + androstenedione or with vehicle.

Morphological measurements taken from nestlings 26 days after hatching showed no

significant treatment effect. In 2007, nestlings with more yolk androgens had lower

hatching mass and higher hematocrit levels 15 days after hatching than nestlings with less

yolk androgens, but the effects disappeared by day 26. In 2008, there were no effects of

androgens on mass or hematocrit. Because the results were not consistent between years,

and because field year had a significant effect on many morphological and blood

measurements, androgens may be linked to the environmental conditions. Over two field

seasons, control clutches and clutches given the largest dosage of androgens had the

highest number of nestlings hatch and fledge. The survival rate from a clutch was

affected differently depending on the amount of exogenous androgens injected into the

eggs. A study by Sockman and Schwabl (2000) supports the anomaly of lowered

survival as a function of exogenous androgens, but their nestlings died mostly after

hatching and the kestrels in my study died before hatching.

Introduction

Unlike the offspring of mammals, whose development is assisted by maternally derived

nutrients while still in the womb, offspring of egg-laying species have limited access to

maternal resources during development. Once an egg is laid, its development proceeds

without further nutritional or hormonal influence from the mother. However, in addition

to their genetic contribution, mothers do have some influence on their eggs' phenotype

through variation in egg number and size, laying date, incubation pattern, and through the

deposition of maternal hormones, such as anabolic steroids, in the yolk, which can affect

offspring survival and consequently the mother's reproductive fitness (Mousseau and Fox

1998a, b). The production of healthy offspring can be influenced by egg-laying parents

in multiple ways, such as Julian date of clutch initiation (Beukeboom et al. 1988, Perrins

1970) and incubation patterns (Aparicio 1998, Wiebe et al. 1998, Sockman and Schwabl

1998). Females also may invest different levels of energy into the eggs such as yolk

androgens (Cunningham and Russell 2000, Sasvári et al. 2004). Maternal effects can

alter offspring phenotype and therefore influence the female's fitness.

Yolk Androgens

Yolk androgens affect early development in many egg-laying vertebrates, including fish

(McCormick 1999), reptiles (Janzen et al. 1998), and birds (Sockman and Schwabl 2000).

These effects may be expressed through changes in offspring survival, hatching timing,

size, secondary sexual characteristics, and immunity. In American kestrels (*Falco

sparverius*), the egg contains nutritional and hormonal resources from the mother

(Newton 1979). Some of these resources are yolk androgens, such as testosterone (T)

and androstenedione (A_4), which are anabolic steroids that affect the phenotypic development of muscles, sexual characteristics, immunity, and behavior (Gil 2003, Navara and Mendonça 2008).

Androgen levels in offspring can be affected by parental condition, weather, and food availability (Sasvári et al. 2004). Avian females deposit variable amounts of androgens in eggs depending on the season, environment, and interactions with both male and female conspecifics. Moreover, increased interactions with aggressive conspecific females may result in increased androgen levels in other adult females, which may result in higher levels of yolk androgens in the eggs (Whittingham and Schwabl 2002, Pilz and Smith 2004; but see Verboven et al. 2005). The amount of androgens a female produces may limit her ability to manipulate androgen levels in eggs. On the other hand, androgen deposition may be a passive process, which simply represents the levels of androgens a female had during the laying process (Verboven et al. 2003).

The pattern of within-clutch variation of yolk androgen levels in birds has been shown to increase sequentially, decrease sequentially, or to have no specific pattern, depending on the species. For example, yolk T increases with each egg laid in canaries (*Serinus canaria,* Schwabl 1993), black-headed gulls (*Larus ridibundus,* Groothuis and Schwabl 2002), and red-winged blackbirds (*Agelaius phoeniceus,* Lipar and Ketterson 2000). By contrast, yolk T levels decrease sequentially in zebra finches (*Taeniopygia guttata,* Gil et al. 2004) and American coots (*Fulica americana*, Reed and Vleck 2001), whereas tree swallows (*Tachycineta bicolor*) show no sequential pattern in yolk androgen deposition (Whittingham and Schwabl 2002). Thus, androgen deposition patterns are extremely variable.

In addition to variability within clutches, patterns of yolk androgen deposition can be found among clutches in response to environmental conditions. For example, female American coots breeding in higher population densities have relatively higher levels of yolk androgens than populations with lower densities (Reed and Vleck 2001). Nest location may also influence yolk androgen levels. For example, clutches of black-headed gulls produced along the edges of nesting areas have higher levels of yolk androgens than those centrally located (Groothuis and Schwabl 2002).

Depending on parental quality, yolk androgen deposition may be influenced by mate preference or by the offspring's sex (Rutstein et al. 2004). Females can produce eggs with higher levels of yolk androgens if they mate with more attractive males, suggesting the costly production of yolk androgens is worth the extra energy to assist in the development of genetically superior offspring (Gil et al. 1999). On the other hand, it has been hypothesized that females can compensate for poor quality males by increasing the levels of yolk androgens (Michl et al. 2004). Preferential treatment can also occur within the clutch; females can provide more or less resources depending on the offspring sex (Gilbert et al. 2005). Although we may not understand what influences the female to alter the androgen deposition, females are able to adjust the levels of these anabolic steroids.

Effects of Yolk Androgens

Although variation in the amount of yolk androgens and the pattern of androgen deposition within a clutch may influence offspring survival, the direction of the effect differs among species. Studies have found both increases and decreases in survival

attributable to yolk androgens during development (Sasvári et al. 2006). One way androgens may increase offspring survival is to help eggs survive cold temperatures (Sockman and Schwabl 1998). Conversely, elevated yolk androgens may reduce offspring survival by lowering hatching success (Navara et al. 2005). Parental condition in Tawny owls (*Strix aluco*) is inversely correlated with the amount of yolk T in eggs laid later in a sequence, and with offspring survival (Sasvári et al. 2004). In American kestrels, offspring survival suffers when the first-laid eggs, which normally contain the lowest amount of androgens, are manipulated to contain the high yolk androgen levels normally found in eggs laid later in the sequence (Sockman and Schwabl 2000). Androgens can also be indirectly associated with survival. For example, Guira cuckoos (*Guira guira*) increase A_4 levels sequentially. Because first-laid eggs are most likely to be ejected from the communal nest, lowered androgen levels in those eggs may reduce investment loss (Cariello et al. 2006). Despite many examples in which yolk androgens influences offspring survival, in some species yolk androgen levels do not affect survival at all (Eising et al. 2001).

Besides influencing offspring survival, at least in some cases, yolk androgens alter inter-egg hatching time and hatching order. In black-headed gulls, late-laid eggs with higher levels of yolk androgens hatch sooner than earlier laid eggs with lower yolk androgen levels (Eising et al. 2001). Although American kestrels eggs increase yolk androgen deposition with laying order, the eggs usually hatch in the same order as they are laid but within four days of each other. This is a shorter time period than the duration of egg laying, in which 72 hours can elapse between successive ovipositions, with an average of four eggs per clutch (Balgooyen 1976, Wiebe et al. 1998). However, it is

important to note that the effects of yolk androgens on hatching may be confounded by uneven incubation by parents, because kestrels have a small body size relative to their egg size (Newton 1979, Bortolotti and Wiebe 1993).

Yolk androgens affect the size and strength of avian embryos and nestlings during development. Some nestlings grow larger overall with higher levels of yolk androgens (Pilz and Smith 2004). Yolk T increases the size and strength of the hatching muscle, which assists the juvenile as it breaks out of its shell and initiates begging behavior (Lipar and Ketterson 2000, von Engelhardt et al. 2006). Also, an increase in yolk androgens increases growth rate during development (Groothuis et al. 2005a). For instance, in black-headed gulls and Eastern bluebirds (*Sialia sialis*), increased levels of yolk androgens increase the leg length of nestlings (Eising et al. 2001, Navara et al. 2005). Chinese painted quail (*Coturnix chinensis*) chicks show no significant mass increase with higher levels of yolk androgens (Andersson et al. 2004); however, because these chicks are precocial, they do not compete against each other for food provisioned by their parents, unlike altricial chicks.

Although anabolic steroids are often associated with the development of secondary sexual characteristics and behavior in adult birds (Sockman et al. 2004), yolk androgens can affect the development of these characteristics as well. For example, canary nestlings with higher yolk androgen levels rank higher socially as juveniles than those nestlings with less yolk androgens (Schwabl 1993). One secondary sexual characteristic linked to yolk androgen levels is structural asymmetry in digits (Burley and Foster 2004), and symmetry can influence mate choice (Møller 1993, Thornhill and Møller 1998). An increase of yolk androgen levels can also bolster preferred plumage

characteristics (Strasser and Schwabl 2004). Since yolk androgens may influence secondary sexual characteristics, they may influence sexual selection.

Immunosuppression is another known effect of increased yolk androgens (Groothuis et al. 2005a). The increased energetic costs of growth and development that accompany the anabolic effects of elevated yolk androgens may result in reduced resource availability for other physiological mechanisms. For example, androgens decrease the response of T-cells and B-cells to infection (Da Silva 1999). However, the immunosuppressive effects of testosterone are not consistent among all avian species. In adult birds, many species with increased testosterone exhibit decreased antibody and cell-mediated responses (Duffy et al. 2000, Casto et al. 2001, Peters 2000). However, others show no correlation between T levels and immunocompetence (Hasselquist et al. 1999). Moreover, both cell-mediated immunity and humoral immunity in black-headed gull offspring are negatively affected by an increase of yolk androgen levels (Müller et al. 2005). Likewise, Eastern bluebird nestlings with artificially increased levels of androgens in their eggs show lower T-cell immune responses (Navara et al. 2005). Nestling zebra finches exhibit gender specific effects of maternal androgens. For instance, an artificial increase of T weakens the immune system of males and strengthens the immune system of females (Rutkoska et al. 2007). In nestling Chinese painted quail, a negative correlation exists between yolk T levels and immunocompetence (Andersson et al. 2004). Immunoresponsiveness of nestlings is influenced by yolk androgen deposition but with varying results.

A Developmental Query Concerning American Kestrels

A study on American kestrels by Sockman and Schwabl (2000) found a decrease in survival to fledging with higher levels of yolk androgens, which is inconsistent with the results of studies of other avian species with similar nesting strategies (Sasvári et al. 2004). A goal of my study was to replicate many aspects of Sockman and Schwabl's investigation, as well as to expand the scope of the investigation, in an attempt to gain a better understanding of the effects of yolk androgens on American kestrel nestlings. Sockman and Schwabl (2000) chose a treatment dosage of androgens that correspond with the highest levels found in natural clutches, and they applied this dosage to first-laid eggs. The effect in their study was decreased overall survival of nestlings. In the present study I use the same dosages as Sockman and Schwabl (2000), as well lower dosages, to determine whether a broader range of yolk androgen levels provides a clearer picture of the effects yolk androgens have on this species.

Hypothesis

The aim of my study was to gain a better understanding of the effects yolk androgens have on nestlings, and the potential effects mothers can have on the phenotypes of their offspring through androgens in the eggs. To that end, I tested the hypothesis that yolk androgens influence physiological and morphological characteristics of nestlings. I predicted that the addition of yolk androgens would result in (1) increased size and growth rate of nestlings, (2) enhanced sexual characteristics of symmetry and subterminal tail-band length, (3) reduced immunoresponsiveness, and (4) decreased survival rate as found by Sockman and Schwabl (2000). To determine whether yolk androgens affect

these characteristics, I manipulated the amounts of the androgens testosterone and androstenedione in American kestrel eggs.

Methods

Study site

American kestrels (*Falco sparverius*) in Ada and Canyon counties, Idaho (43° 33' 40" N, 116° 28' 25" W) nested in artificial nest boxes (21 cm x 21 cm x 46 cm) placed approximately 2.5 m high on telephone poles along roadways. Box locations were in two general areas, one group in east Boise, ID and another near Meridian, ID. Egg laying began in April, and nests were active through July. Some of the boxes have been established for as long as nine years, whereas others were erected just prior to the 2007 and 2008 field seasons. The nest boxes were situated in areas that included natural shrub-steppe, feedlot ranch , agriculture (wheat, corn, barley, and hay), and suburban areas.

Androgen Treatments

One of three androgen dose treatments was used for all eggs in a given clutch. In each treatment the androgens were dissolved in 50 μL of sesame oil (Sockman and Schwabl 2000), which I refer to as the "vehicle." In the 2007 breeding season, the three treatment levels included: the control (i.e., vehicle only), a low dose of androgens (0.025 μg of T and 1 μg of A_4), and a medium dose of androgens (0.075 μg of T and 3 μg of A_4). In the 2008 breeding season, the level used by Sockman and Scwhabl (2000), which was 0.1 μg of T and 4 μg of A_4, replaced the low dose of androgens. This dosage level is referred to

as the high androgen dose. I was blind to the dosage level I injected into the eggs of a clutch until after completion of all measurements.

I began visiting nest boxes weekly in early March, which corresponded with the initiation of egg laying (Balgooyen 1976). Androgen injections were administered between 0600 and 1300. Clutches were randomly assigned either to a control or to treatment groups (low or medium dose in 2007, medium or high dose in 2008). The first clutch was randomly assigned one of the three treatments. The next clutch was randomly given one of the two remaining treatments, and the last treatment was given to the third clutch. That process was repeated for the next three clutches and continued through the season to evenly stratify the dosage levels temporally. Once a clutch had three eggs, the eggs received an injection with the assigned treatment, and the nest was checked daily for new eggs that were injected until the clutch was complete. To lower the risk of nest abandonment, the clutch was not disturbed during incubation. A small amount of cyanoacrylic glue (in 2007) or New Skin's "Liquid Bandage" © (in 2008) was used to seal the hole left by the injection. All methods were approved in advance by the Boise State University IACUC (protocol number 006-05-011).

Nestling Identification

Because incubation in American kestrels extends approximately 27 days from when the penultimate egg is laid (Porter and Wiemeyer 1972), after the initial injections, I began to check the clutches for hatched nestlings after 25 days of incubation. Nests in which eggs were cold for over a week were considered to be abandoned and were excluded from the dataset. Once an egg began to pip, or when the nestling broke out of its shell, I used a

non-toxic marker to color hatchlings on the top of their heads. A unique color, picked

randomly using a Latin square design, was applied to each nestling (Sockman and

Schwabl 2000). Because the markings faded over time, I revisited the nestlings every

three to four days and reapplied the markings. Offspring were banded at 15 days of age

with numbered aluminum bands supplied by the USGS.

Survival

A nest was deemed successful if at least one offspring survived until day 26. The number

of nestlings that survived to day 26 per number of eggs laid in each clutch determined the

survival rate of the clutch.

Growth

Hatching date in 2007 was determined following Griggs and Steenhof (1993). The hatch

day of the oldest nestling was considered to be the hatch day (i.e., day 1) for the entire

clutch. The nestlings were visited every three to four days, up to day 26, to obtain growth

measurements: body mass, tarsus length, wing chord, and tail length. To improve the

accuracy of the hatch day, in 2008 I visited nest boxes repeatedly to determine the

specific hatch day of each individual nestling. Instead of regularly measuring

morphological measurements through the entire nestling stage, I focused on the initial

post-hatching days to examine the effects of androgens earlier in development.

Measurements of mass and tarsi were taken for each nestling on their respective days 1,

2, 3, 4, and 5. As in 2007, mass, tarsus length, wing chord, and tail length were measured

for all nestlings on day 26.

Skeletal Growth

To assess skeletal growth, bone alkaline phosphatase (BALP) was measured from blood

samples collected on day 15 and 26 (Brixen et al. 1989, Farley and Baylink 1995).

Measurement of this isoenzyme provides a more accurate estimate of the rate of bone

formation than overall alkaline phosphatase (ALP) (Brixen et al. 1989, Farley and

Baylink 1995). The amount of serum BALP is directly related to osteoblast production

and bone genesis (Hoffman et al. 1999). Moreover, BALP has less daily variation than

other enzymes, and often it is the isoemzyme of greatest abundance in younger organisms

(Sanecki et al. 1993).

BALP is difficult to distinguish from the other isoenzymes because of its

structural similarities with other ALP isoenzymes (Fishman 1990, Hoffman et. al 1999,

Moss 1982). Therefore, to isolate the BALP isoenzyme, I used wheat-germ lectin (WGL)

to precipitate out BALP (Harris 1989, Rosalki and Foo 1984, Lis and Sharon 1973).

Wheat-germ lectin from *Triticum vulgaris* has been used successfully to precipitate

BALP out of serum in humans, birds, rats, and dogs (Behr and Barnert 1986, Sanecki et

al. 1993, Ladizesky et al. 2003, Smits et al. 2007). Although WGL precipitates out

BALP and liver ALP, the optimal concentration of WGL precipitates the maximum

amount of BALP and only a negligible amount of liver ALP (Hoffman et al. 1994,

Weidmeyer et al. 1999, Rosalki and Foo 1984, Behr and Barnert 1986, Woitge et al.

1996).

I used a combination of published methodologies to measure BALP. Plasma from

American kestrels was stored at -20° C for one year (2007 samples) or a few months

(2008 samples) prior to BALP analysis; freezing does not alter the structure of the

enzyme ALP (Terefe et al. 2004). Wheat-germ lectin (Sigma Aldrich, L9640) was used to precipitate out BALP (Rosalki and Foo 1984, Smits et al. 2007). The nestling plasma samples from 2007 were pooled together to make one sample for each clutch, whereas the nestling plasma samples from 2008 were analyzed individually. For both years, each plasma sample was divided into two microcentrifuge tubes containing 50 μL each. Fifty μL of distilled water were added to one of the plasma tubes, and 50 μL of WGL (5 g/L in distilled water) were added to the other tube (Behr and Barnert 1986). The tubes were then incubated for 30 min at 37° C to allow the BALP to precipitate out. All tubes were centrifuged for 2 min at 13,000 rpm. The supernatant was decanted and analyzed for all alkaline phosphatase. A glycine buffer containing 25 mMol glycine and 50 μMol $MgCl_2$ was altered to a pH of 10.0 with NaOH (Vertommen et al. 1980, Kuettener et al. 1971). Five mMol of *p*-nitrophenyl phosphate (PNPP) were then added to the buffer. The decanted plasma (20μL) and 1 mL of the buffer/PNPP mixture were analyzed in a spectrophotometer (Lambda 35 Perkin Elmer) at 405 nm in absorbance/min. PNPP (BioChemika) was converted into the yellow nitrophenol when mixed with the alkaline phosphatase found in the plasma (Vertommen et. al 1980).

The units (U) of BALP were determined by finding the absorbance/min of the sample on the slope-of-progress curve. Using the molar extinction coefficient of PNPP ($0.0186 \ \mu M^{-1} cm^{-1}$; Schlatter et al. 2002) and the dilution factor of 100, the U/L were determined by the following equation:

$$U/L = D \ Abs_{405} / (L \ \varepsilon),$$

where U/L = (μmole/min)*L of plasma, D is the dilution factor, Abs_{405} = Absorbance per minute, L = length of cuvette (i.e., 1 cm), and ε = molar extinction coefficient of 0.0185 $\mu M^{-1} cm^{-1}$. The difference between the samples with and without the WGL is the precipitated bone alkaline phosphatase levels.

Hematocrit

Hematocrit levels provide a general indication of body condition (Morton 1994). Blood samples were collected from the brachial vein in micro-hematocrit capillary tubes on nestling days 15 and 26 in 2007 and 2008 to determine the hematocrit levels. The micro-hematocrit tubes were centrifuged on a HemataSTAT-II (Separation Technology, Inc.), and the hematocrit level was calculated from the ratio of packed cell volume to plasma volume. Plasma was removed and stored in microcentrifuge tubes at -20˚ C.

Secondary Sexual Characteristics

I measured two secondary sexual characteristics of juvenile kestrels: subterminal tail band length and fluctuating asymmetry of the wing chords and tarsi (Wiehn 1997). The bird's sixth tail feather from the left was selected to measure the length of the subterminal black tail band at the shaft. Both right and left wing chords and tarsi were measured.

Immunoresponsiveness and Heterophil to Lymphocyte Ratio

To assess cell-mediated immunity in the kestrels, phytohemagglutinin (PHA) was used to elicit a response of T cell lymphocytes (Goto et al. 1978). In 2007, PHA was injected into the right patagium of sample kestrels 25 days after hatching. Before making the

injection, I marked the patagium with a non-toxic black marker and then measured the thickness of this location twice with a digital micrometer. I then injected the kestrel with 50 μg of PHA (Sigma) dissolved in 50 μL of phosphate buffered saline (PBS) (Smits et al. 1999) at the location of the marking. Approximately 24 hr later the patagial thickness was measured again to determine the amount of swelling. The extent of the cell-mediated response was indicated by the amount of swelling due to the mitogenic effects of the PHA on T lymphocytes. This reflects the bird's immunoresponsiveness to a potential harmful pathogen (Lis and Sharon 1973).

Heterophils are associated with responses to bacterial infections, whereas lymphocytes are related to chronic viral infections. The ratio between heterophils and lymphocytes can be used as a body condition index, with an increase in the heterophil to lymphocyte (H/L) ratio indicating a decrease in general body condition (Gross and Siegel 1983). In the present study, blood from the brachial vein was smeared to make monolayer slides to determine the H/L ratios. Smears were fixed with a solution of 80% methanol and 20% distilled water for 1 min. Slides were stained with Wright-Giemsa quick stain for 5 min, followed by 5 min in Volu-Sol's hematology buffer and a 10 sec rinse (method for non-mammalian cells from Volu-Sol). The heterophils and lymphocytes were then counted by microscope (100x power, oil immersion), and 100 white blood cells were counted by Ms. Lynda Leppert, of the Alaska Sea Life Center, to determine the H/L ratio (Tella et al. 2000, Hawkey and Dennett 1989). Ms. Leppert was blind to the treatment groups.

Data Analysis

Statistical tests were conducted using SAS 9.1 and JMPIN (SAS Institute, Cary, NC).

The experimental unit for analyses was clutch. As well as examining each year

separately, data across years were pooled when I found no statistical effect of year for the

control and medium treatment (i.e., the manipulations used in both years) despite minor

differences in the way clutches were treated each year. In such cases I assumed that the

treatments used for only one field season (low and high) also had no year effect.

Considering this assumption, years were combined only if the effect of year yielded a $P >$

0.10 to be conservative. However, if treatment effects were statistically different

between years ($P < 0.10$), the data were not pooled. If a treatment effect was found, then

I used Proc Mixed or Proc Genmod post-hoc tests (differences of least square means) to

compare treatments. A Bonferroni correction was not applied when comparing

treatments in these pair-wise tests so as not to be overly conservative with field results

(Nakagawa 2004).

      Proc Mixed was used to determine the effect of treatment on size, secondary

sexual characteristics, skeletal growth, hematocrit, H/L ratio, and immunoresponsiveness.

Proc Mixed was also used to find any difference in variation for each treatment and to

determine if there was an effect of year for the variables.

      Asymmetry in the wing chords and tarsi was analyzed using Kolmogorov

Smirnov tests. The absolute value of the difference between the left and right side was

calculated and nestlings in a clutch were averaged to get one clutch value. Each

treatment was compared to each other, and then each sex was looked at individually.

Nest success was analyzed using a χ^2 Goodness-of–Fit test by treatment. The Goodness-of–Fit test was also used to determine if location altered nest success, and to determine if treatment affected the survival of each sex differently. Because of low sample size, Fisher's Exact Test was used to evaluate the effect of location on nest success in 2008. A χ^2 Goodness-of–Fit test was used to determine if there was an effect of year on nest success.

Survival rate was determined by comparing the number of nestlings that survived to day 26 per number of eggs laid. The survival rate was analyzed with a Poisson regression with a log transformation in Proc GENMOD to determine if there was an effect of treatment or year.

Growth rate was analyzed by fitting every nestling with a nonlinear Gompertz growth curve fitted on JMP. Then the parameters (a_0, b_0, b_1) had low correlation (correlation coefficients less than .66) and were analyzed Proc Mixed (Sockman et al. 2008):

Gompertz growth curve
$$\text{Mass} = a_0\exp(-b_0 b_1{}^{\text{age}})$$

Where a_0 = the asymptote of the final mass
b_0 = initial mass at hatching
b_1 = inflection point
Age = number of days after hatching

Results

<u>Survival</u>

Nest success was determined by the survival of at least one nestling 26 days after hatching; the number of clutches that were successful did not differ significantly due to the addition of yolk androgens. See Table 1.1 for a summary of androgen treatments for

Table 1.1. Results of control injections or injection of low, medium, or high levels of androgen into American kestrel eggs in 2007 and 2008. The number of clutches in a given category are shown. A successful clutch had at least one nestling survive to day 26 after hatching.

| Year | Success | Treatment | | | | Grand Total |
		Control	Low	Medium	High	
2007	Fail	4	11	8		23
	Succeed	11	6	8		25
	2007 Total	15	17	16		48
2008	Fail	4		4	2	10
	Succeed	5		6	8	19
	2008 Total	9		10	10	29
	Grand Total	24	17	26	10	77

the clutches. The effect of androgen dosage on nest success was not statistically

significant in 2007 (χ_2^2 [N = 48] = 4.66, P = 0.09) or in 2008 (χ_2^2 [N = 29] = 1.46, P =

0.48). Androgens affected development of male and female chicks similarly (2007: χ_1^2

[N = 49] = 1.20, P = 0.55; 2008: χ_2^2 [N = 48] = 4.76, P = 0.09). Location affected

success in 2007, and Boise nests were more successful than Meridian nests (χ_1^2 [N = 48]

= 4.41, P = 0.04). No difference was found in 2008 (Fisher's exact test, P = 0.53). Nest

success did not differ significantly between years (χ_1^2 [N = 77] = 2.26, P = 0.13).

Survival rate, the ratio of the number of nestlings that fledged to the number of eggs laid,

was significantly affected by yolk androgen dosage in 2007 (χ_2^2 = 15.86, P < 0.001).

Control clutches had a significantly higher number of fledged nestlings per clutch than

low and medium dosage clutches (both P < 0.01). However, the low dosage clutches and

medium dosage clutches were not significantly different from each other (P = 0.19). In

2008, yolk androgen dosage had a significant overall effect on survival rate (χ_2^2 = 8.55, P

= 0.01). Survival rate in the high dosage treatment was higher than in both the control

and medium dosages (both P < 0.05); however, the control and medium dosages were not

significantly different from each other (P = 0.53). Because year had no effect on the

survival rate of the treatment groups (χ_1^2 = 0.01, P = 0.92), the two field seasons were

combined for analysis. Androgen dosage had a significant overall effect on survival rate

(χ_3^2 = 22.37, P < 0.0001). The control and the high androgen dose had the greatest

number of surviving nestlings, whereas, the intermediate dosage levels had fewer
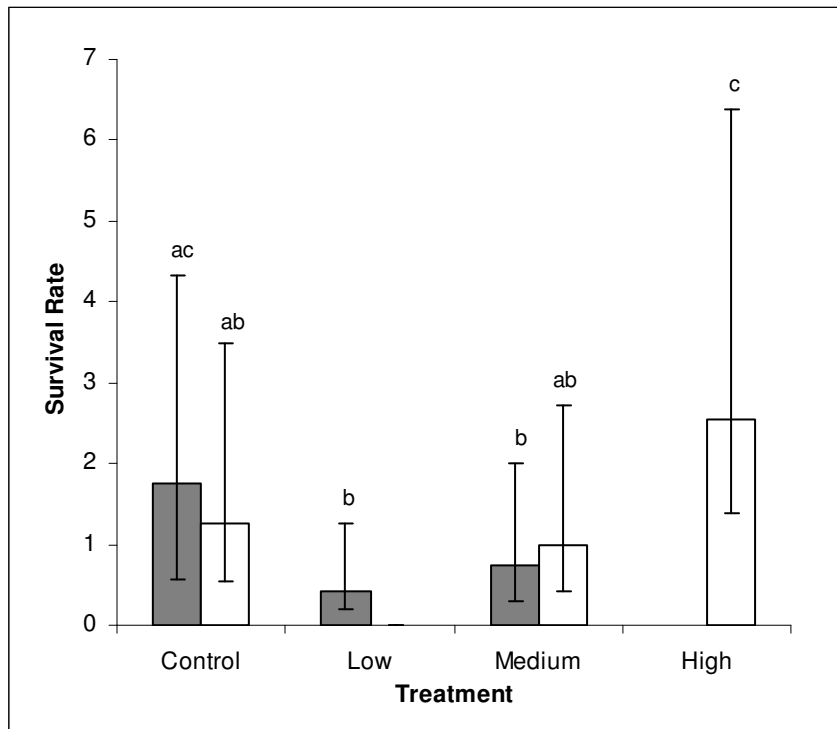
nestlings survive in a clutch (Figure 1.1).

Figure 1.1. Mean nestlings per clutch (taken from clutches of 5) that lived to day 26 from the total number of eggs laid in both field seasons (N = 77). Lines span 95% confidence intervals, and the log-transformed data were back-transformed. Different letters denote a significant difference among treatments. 2007: shaded bars; 2008: open bars.

Size and Growth Rate

Among the variables measured on day 26 (mass, left and right tarsus, tail length, left and right wing chord), none showed significant treatment effects as a result of androgen injections into the eggs. The addition of androgens did not affect nestling mass on day 26 (2007: $F_{2,24} = 0.45$, $P = 0.64$; 2008: $F_{2,29} = 0.01$, $P = 0.99$), nor did it have a significant effect on the length of either tarsus (right tarsus - 2007: $F_{2,24} = 1.14$, $P = 0.34$; 2008: $F_{2,29} = 0.04$, $P = 0.96$; left tarsus - 2007: $F_{2,24} = 1.24$, $P = 0.31$; 2008: $F_{2,29} = 0.20$, $P = 0.82$). Because no significant differences were found between 2007 and 2008 for mass on day 26, right tarsus, and left tarsus, data from both years were pooled. When this was done there still was no significant effect of treatment (mass: $F_{3,37.1} = 0.14$, $P = 0.93$; right tarsus: $F_{3,66.2} = 0.26$, $P = 0.85$; left tarsus: $F_{3,57.4} = 0.41$, $P = 0.75$).

Morphological characteristics of length were also not affected by the amount of androgens given during development. The tail length of nestlings on day 26 was not significantly different among treatment groups (2007: $F_{2,24} = 1.11$, $P = 0.35$; 2008: $F_{2,29} = 0.66$, $P = 0.52$). Wing chords were also measured to determine if T and A_4 affected their development, but measurements taken 26 days after hatching suggested that there were no effects (right wing chord - 2007: $F_{2,24} = 1.37$, $P = 0.27$, 2008: $F_{2,29} = 0.03$, $P = 0.97$; left wing chord - 2007: $F_{2,23} = 1.22$, $P = 0.31$, 2008: $F_{2,29} = 0.05$, $P = 0.95$). Tail length ($F_{1,77.7} = 32.51$, $P < 0.001$) and right wing chord ($F_{1,78.7} = 4.90$, $P = 0.029$) measurements both decreased significantly from 2007 to 2008, so the data were not combined. Also, left wing chords were marginally lower in 2008 ($F_{1,82.5} = 03.76$, $P = 0.056$), so they were conservatively not combined either.

Measurements taken at 26 days occurred nearly two months after eggs were injected with androgens, which may have overlooked the effect of androgens early in development. To gain a better understanding of early size and growth rate, the periodic mass measurements taken through the nestling period were fitted to a Gompertz nonlinear growth curve with parameters for the mass asymptote (a_1: the final adult mass), the growth rate inflection (b_1: the shape of the growth curve), and the initial mass (b_0: the mass at hatching). Looking at each year separately, an effect of treatment on the final adult mass (a_1) was not found in either year (2007: $F_{2,24} = 0.76$, $P = 0.48$; 2008: $F_{2,29} = 0.14$, $P = 0.87$). Likewise, the pattern of growth over time (b_1) was not significantly affected by androgen levels during development (2007: $F_{2,24} = 1.04$, $P = 0.37$; 2008: $F_{2,29} = 0.16$, $P = 0.85$). In 2007, there was a significant overall effect of androgen levels in eggs on initial hatching mass, b_0 (Figure 1.2, $F_{2,24} = 3.66$, $P = 0.041$). Specifically, control eggs produced initially larger nestlings than eggs injected with the medium dosage of androgens ($t_{24} = 2.70$, $P = 0.013$). However, 2008 did not show an effect of T and A_4 on the hatching mass, b_0 ($F_{2,29} = 0.44$, $P = 0.65$). The three parameters found by the Gompertz nonlinear growth curve were all significantly different between 2007 and 2008, so the data were not combined. Relative to the 2007 values, both final adult mass and growth curve increased in 2008, whereas hatching mass decreased (a_1: $F_{1,48.8} = 4.31$, $P = 0.0433$, b_1: $F_{1,83.6} = 49.22$, $P < 0.0001$, b_0: $F_{1,80.1} = 12.12$, $P = 0.0008$).

Skeletal Growth

After analyzing the plasma samples, I found no treatment effects on the levels of BALP on day 15 (2007: $F_{2,19} = 0.50$, $P = 0.61$; 2008: $F_{2,28} = 1.27$, $P = 0.30$). Day 26 blood
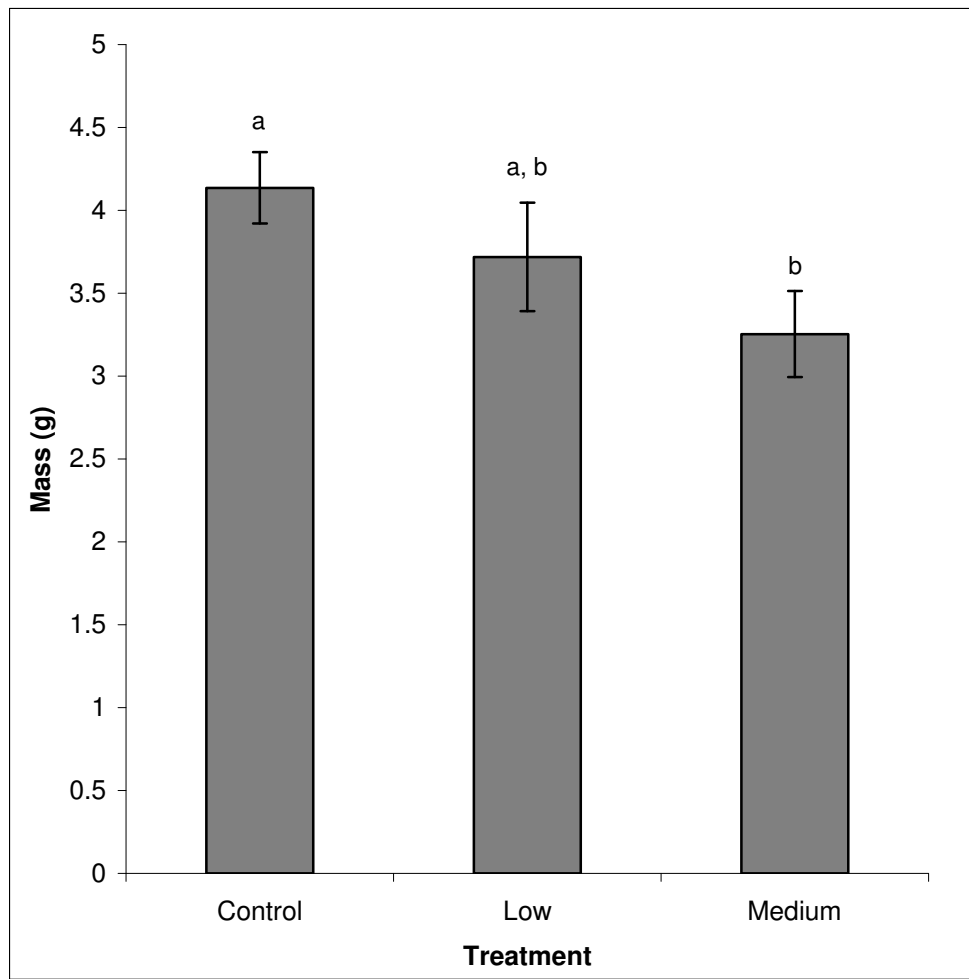
Figure 1.2. The nonlinear Gompertz growth curve parameter for mean hatching mass ($\pm$ SE) in 2007 (N = 25). Different letters denote a significant difference among treatments. The control group received no exogenous androgens, the low dosage group received 0.025 μg of T and 1 μg of A_4, and the medium dosage group received 0.075 mg of T and 3 mg of A_4.

samples also showed no effect of androgens on BALP (2007: $F_{2,19} = 0.86$, $P = 0.43$; 2008: $F_{2,27} = 1.99$, $P = 0.15$). Because the 2007 and 2008 samples were analyzed differently (2007 nestlings were pooled together into one clutch sample, 2008 each nestling was analyzed separately and averaged), the years were not combined.

Hematocrit

Hematocrit levels decreased significantly as a function of Julian date ($F_{1,24} = 4.89$, $P = 0.04$). Therefore, Julian day was added as a covariate in the model that examined the effect of androgens on hematocrit levels. Controlling for Julian date, there was a significant overall effect of treatment on day 15 hematocrit levels in 2007 (Figure 1.3, $F_{2,24} = 4.82$, $P = 0.02$). Although the control and low androgen dosage treatment did not differ significantly from each other ($t_{24} = 0.39$, $P = 0.70$), both had significantly lower hematocrit levels than the medium androgen dosage treatment (control vs. medium: $t_{24} = -2.81$, $P < 0.01$; low vs. medium: $t_{24} = 2.56$, $P = 0.02$). However, the increased hematocrit levels in the medium androgen dosage were not apparent by day 26 ($F_{2,24} = 0.22$, $P = 0.80$). In 2008 there were no differences in hematocrit levels among treatment groups (day 15: $F_{2,29} = 1.16$, $P = 0.32$; day 26: $F_{2,29} = 2.34$, $P = 0.11$). Hematocrit levels were not significantly different between years ($F_{68.9} = 1.42$, $P = 0.24$; Day 26: $F_{1,78.3} = 0.86$, $P = 0.36$) and when combined had not significant difference by treatment ($F_{3,58} = 2.52$, $P = 0.07$; Day 26: $F_{3,58} = 1.53$, $P = 0.39$).
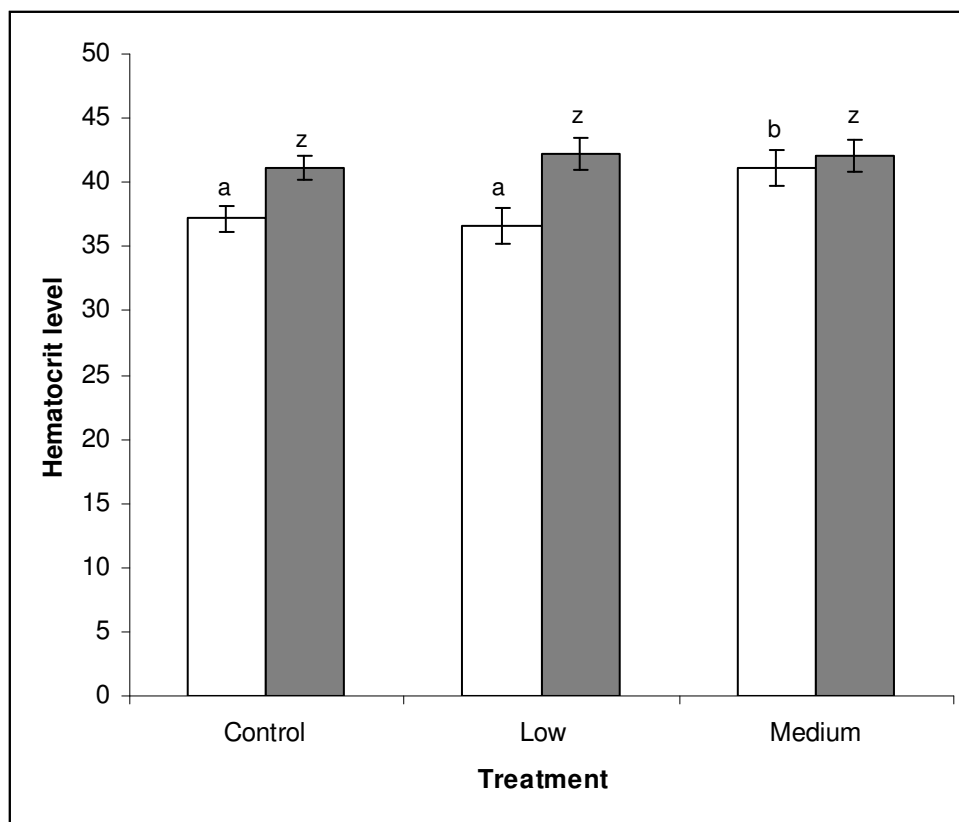
Figure 1.3. Mean hematocrit levels taken from all nestlings in a clutch on the 15th day (open bars) and 26th day (shaded bars) of the oldest nestling in the 2007 field season (N = 25), ± standard error. Different letters denote a significant difference among treatments; however, day 15 and day 26 measurements were not compared to each other within treatment groups. The control group had no androgens injected, the low dosage group received injections of 0.025 μg of T and 1 μg of A_4, and the medium dosage group received injections of 0.075 mg of T and 3 mg of A_4.

Secondary Sexual Characteristics

Overall, no secondary sexual characteristics showed a significant influence of androgens during development. No significant differences were found in the size of the black subterminal tail band among kestrels with different androgen dosages in either year (2007: $F_{2,7} = 0.09$, $P = 0.91$; 2008: $F_{2,9} = 0.37$, $P = 0.70$). There was no effect of year ($F_{1,42} = 1.25$, $P = 0.27$), but the combined dataset still showed no treatment effect ($F_{3,31} = 0.15$, $P = 0.93$). The absolute value of the difference between the left and right measurements of the tarsus and the wing chords analyzed the effects of yolk androgens on asymmetry; androgen injections had no effect on asymmetry in the tarsus and the wing of nestlings on day 26 for either year or sex (Kolmogorov–Smirnov $P > 0.05$ in all cases).

Immunoresponsiveness and Heterophil to Lymphocyte Ratio

Immunoresponsiveness was predicted to decrease with more yolk androgens, but no difference was found among the treatments for PHA in 2007 ($F_{2,22} = 0.68$, $P = 0.52$). Similarly, the addition of yolk androgens did not affect day 15 H/L ratios (2007: $F_{2,22} = 0.09$, $P = 0.91$; 2008: $F_{2,27} = 0.01$, $P = 0.99$) or day 26 H/L ratios (2008: $F_{2,29} = 1.14$, $P = 0.33$).

Discussion

The primary aim of my study was to develop an understanding of the effects of androgens on American kestrel nestlings. Although my study added to the comprehensive picture of yolk androgens effects, the outcomes were far from clear because results differed between years. Yolk androgens are key resources necessary for

development, and they influence factors such as hatchability, growth, blood composition, and possibly symmetry that can be used in mate choice (Møller 1993). The effects of yolk androgens are narrowly defined into specific functions, but research is showing that androgens have a wider range of effects. Certain effects of androgens, such as survival, may not occur in one manner in all situations (Sasvári et al. 2004). In my study, the effects of androgens differed between the two field seasons and may have been influenced by environmental factors such as temperature and precipitation. My results indicate that the addition of exogenous yolk androgens does affect kestrel development, but in a complex way that may be limited to early development as illustrated in the survival rate of the kestrel nestlings.

<u>Survival</u>

Androgens may affect survival by increasing both muscle mass and begging behavior, which would allow offspring with higher androgen levels to receive more food. Those offspring would grow and develop to be superior to offspring given fewer resources (Lipar and Ketterson 2000). The survival rate found in the first year of my study mirrored Sockman and Schwabl's (2000) findings in which yolk androgens decreased survival. However, the results of the second year of my study supported the more general view that androgens enhance survival (Lipar and Ketterson 2000, Pilz et al. 2004, Gil 2003, Groothuis et al. 2005b). Sockman and Schwabl (2000) discovered a decrease in survival in eggs injected with higher amounts of A_4 and T, whereas androgens had little observable impact post-hatch in this study.

One goal of my study was to expand on the Sockman and Schwabl (2000) study using a similar approach. They monitored offspring development after injecting the first-laid egg of a clutch with an androgen level that approximated the higher androgen level typically found in the fourth-laid egg. Only one nestling survived to fledging of the 10 that received the increased androgen treatment. In my experiment, I injected the entire clutch with the same or lower dosage levels, and the clutch, rather than the individual, was the experimental unit in my study. Only four of 101 nestlings (4%) died after hatching in my study: one control, one medium-dose, and two high-dose birds. The percent survival to fledging of birds given exogenous yolk androgens in Sockman and Schwabl's (2000) study (1/10, 10%) differs markedly from the percent survival to fledging of my nestlings (97/101, 96%). It is unclear why this occurred. Sockman and Schwabl (2000) injected only first-laid eggs with androgens whereas I injected all eggs in a clutch; perhaps first-laid eggs are more sensitive to the addition of yolk androgens than are later-laid eggs and thus they succumb disproportionately.

Because natural androgen levels increase with laying order (Sockman and Schwabl 2000), each of the eggs in my study had different amounts of total yolk androgens; however, I assumed the natural androgen deposition was consistent from clutch to clutch (see Sockman and Schwabl 2000). The overall yolk androgen level was increased for all eggs, not just the first-laid egg, and the higher levels could alter effects. Such an effect is not possible to tease out of my data because I injected all of the eggs and did not track laying order. In addition, in 2007 the eggs in my study were exposed to a lower amount of androgens than that used in Sockman and Schwabl (2000), and possibly a threshold occurs beyond which the addition of exogenous androgens becomes

toxic. However, considering only the eggs from 2008 that were injected with the same amount of yolk steroids as those in Sockman and Schwabl (2000), 92% (24/26) survived to leave the nest, which is still a considerably higher fledging rate than that reported in Sockman and Schwabl (2000). Clearly, the addition of exogenous yolk androgens to kestrel eggs does not necessarily produce high levels of mortality.

Although post-hatch effects were minimal, the overall survival rate of offspring that hatched and survived to fledging had contrasting results between years. In 2007 the results supported the notion from Sockman and Schwabl (2000) that exogenous androgens increase mortality, but in 2008 mortality decreased with the addition of androgens. Sockman and Schwabl (2000) did not report any effects on hatching success due to the addition of yolk androgens. However, in my study the low and medium dosages hatched significantly fewer young than the control dosage. Navara et al. (2005) also found a decrease in hatching success with an increase in exogenous yolk T for Eastern bluebird nestlings (*Sialia sialis*), and clutches with the highest dosage of testosterone had barely half the hatching success of the control clutches. The reasons why mortality sometimes increases before or after hatching in response to yolk androgens are unknown and are worthy of investigation.

Not only did the timing of mortality in my study differ from that reported in Sockman and Schwabl (2000), but the overall rate of survival differed between years. In one season exogenous androgens lowered survival rate, whereas in the other season they increased survival rate. The effect of exogenous androgens may be dosage dependent or involve external influences such as environmental conditions. Either way, their impact on survival is more complex than simply increasing or decreasing survival. In Sockman

and Schwabl's (2000) experiment, the first laid egg was injected with androgens, which made the amount of yolk androgens in first and last-laid eggs equivalent, and decreased the survival of the nestling from the first-laid egg. In 2007 I found that the low (0.025 μg of T and 1 μg of A_4) and medium (0.075 μg of T and 3 μg of A_4) androgen levels (both lower than the 0.1 μg of T and 4 μg of A_4 that Sockman and Schwabl used) decreased the number of eggs that hatched and fledged. In 2008, I used the same dosage level as Sockman and Schwabl (2000), which was higher than my previous year's dosages, and the number of nestlings that survived with the high androgen level was higher than both the control and medium treatments used that year. This produces a quandary as to why the control and the highest androgen level had the greatest success compared to the dosages in between. Nestlings from eggs given the lowest or highest levels of yolk androgens had the greatest chance at survival.

In 2007, the controls had the highest level of survival, but in 2008 the high dosage group had the highest survival. This difference may be due to environmental differences between the years that could affect maternal condition and therefore affect androgen deposition. Female condition can modify the amount of yolk androgens deposited (Sasvári et al. 2006), so if the endogenous levels of androgens in the kestrel eggs were different between the years, then the treatment groups would have different total androgen levels depending on the year. Many environmental factors could have caused a shift in endogenous yolk androgen levels. For instance, monthly precipitation varied, with more precipitation in February in 2007 (1.27 inches in 2007, 0.57 inches in 2008) and more precipitation in March in 2008 (0.32 inches in 2007, 1.21 inches in 2008; from the National Weather Forecast Office's Preliminary Local Climatological Data for

Boise). These months are just prior to the breeding season and the effects of earlier or later rain could initiate germination of plants at different times (Seyfried et al. 2001). The earlier rains in 2007 may have increased the plant biomass and therefore increased prey availability (Murphy 1992). The amount of food available before breeding has been shown to affect maternal androgen deposition (Gasparini at al. 2007), egg mass (Ardia et al. 2006), and hatching success (Valkama et al. 2002). Another difference between the two field seasons was the daily minimum temperature, although Wiebe (2001) did not find differences in survival rate due to temperature changes in cavity nesters.

The differing results between years may be explained by a dosage dependent effect of androgens in conjunction with different external conditions. That is, the effect of yolk androgens on certain physiological characteristics, may reduce survival when combined with one set of environmental conditions, but enhance it when subjected to a different set of conditions. The effects of yolk androgens on American kestrel nestling survival rates are complex, and the amount of androgens deposited can have a wide array of costs and benefits that must be analyzed further to better understand this phenomenon.

Size and Growth Rate

The addition of yolk A_4 and T has repeatedly been shown to increase size and strength in nestlings in other avian species (Pilz et al. 2004, Lipar and Ketterson 2000, von Engelhardt et al. 2006, Schwabl et al. 2007; but see Tobler et al. 2007), although this is not what I found in my study of kestrels. Exogenous androgens either had no effect on growth or decreased the mass. In 2007, hatching mass was lower in the medium androgen treatment group than in the control group. Sockman and Schwabl (2000) found

no initial difference in mass, but androgen treated groups had a lower mass from 10 to 20 days of age, after which they stopped measurements.

Metabolic function may explain lower hatching mass of the 2007 nestlings with increased androgens. Testosterone can increase the basal metabolic rate in some species of birds (Buchanan et al. 2001). One hypothesis to explain lowered mass from an increase in yolk androgens is that elevations in metabolic rate and oxidative stress created by increased levels of T can impair mass gain when food availability is low in the first few days after hatching. However, if food is plentiful, the costs of increased metabolic rate and oxidative stress may be overcome by the anabolic effects of testosterone resulting in increased growth (Royle et al. 2001, Sockman et al. 2006). For example, the resting metabolic rate of 14 to 15 day old zebra finch nestlings was increased for those that had artificial increases in yolk androgens, yet the nestlings with increased androgens did not show increased growth rate (Tobler et al. 2007). The results from this field study could reflect conditional responses to exogenous androgens due to varying circumstances.

Rapid growth and shorter development times can be advantageous if offspring are in an open cup nest that can be depredated easily (Bosque and Bosque 1995). However, because kestrels are cavity nesters, their nestlings may be relatively safe from predators, at least when compared to open cup nesters. Thus, the potential cost of lowered hatching mass in chicks from androgen-injected eggs may not be a major selective force in kestrels. Androgens are correlated with increased predation and decreased incubation length, suggesting that androgens can increase the rate of embryonic development (Martin and Schwabl 2008, Schwabl et al. 2007). However, the growth rate in American

kestrel nestlings may be less sensitive to androgens than open cup nesters, which would explain the lack a treatment effect in growth rate in either year of the study. An increase of growth rate allowing a nestling to reach its asymptotic adult mass more rapidly would not be as critical to kestrels as it is to many other more easily depredated species.

The effect of exogenous androgens on size measurements showed no long-term consequences for growth, so size may not be affected by androgens or, more likely, differences in size may disappear in the absence of sustained elevated androgen levels as individuals near fledging. Tarsus length, wing chord length, and body mass measured 26 days after hatching showed no significant differences among treatment groups. Initial effects of androgens seen in other studies, such as increased neck muscle mass, greater overall body mass, or the lowered mass of hatchlings observed in this study, have been associated with increased yolk androgens (Navara and Mendonça 2008, Pilz et al. 2004), and the lack of differences after 26 days were due to the effects of other factors such as parenting and the environment (Lindström 1999). The nestlings exposed to higher yolk androgen levels gained mass to match the mass of control birds by day 26. Even though nestlings with higher levels of exogenous yolk androgens initially were smaller in mass in one year, the lack of difference at 26 days suggests that yolk androgen level did not have a long-term influence.

Some morphological measurements may have been affected by external conditions that resulted in variation between years. Tail length, wing chord, hatching mass, and growth rate data were each different from 2007 to 2008, so these were not combined. The final growth asymptote increased as well as the growth inflection point, which yielded a more smooth growth rate as opposed to an initial sharp increase in

growth the first week of development that leveled out to the adult mass. Hatching mass,

tail length, and wing chord all were lower in 2007 than in 2008. Some external factor(s)

may have influenced development for these measurements, but those external factors

apparently did not create a year difference for day 26 mass measurements of the left and

right tarsus. The highest dosage was not tested in both years, and because many variables

had different results between the years, then the highest dosage may not have had the

survival success it had in 2008 if it was tested in 2007.   Unknown differences affected

the results between years, but only for some variables. The variables affected may

provide clues to the difference between the years; however, the environmental differences

between years were not analyzed in my study, so the subsequent suggestions are

speculative.

Difference between field seasons, such as the decrease in temperature from 2007

to 2008 (see Figure 1.4), may explain why there are inconsistent results from year to year.

For instance, hatching mass was significantly different among treatment groups in 2007,

but not in 2008. It is possible that the high dosage was high enough to have an effect on

hatching in the colder 2008 year. This dosage was not used in the 2007 field season

because I wanted to explore the effects of smaller androgen increases than those used by

Sockman and Schwabl (2000). Temperature can alter the size of nestlings where an

increase in temperature can decrease mass possibly due to water loss (Murphy 1985).

The first field season was warmer than the second field season, and that may have

affected the local semiarid plants by increasing evaporation in an already dry climate and

shortening the growing season, which could affect the prey population (Fischer and

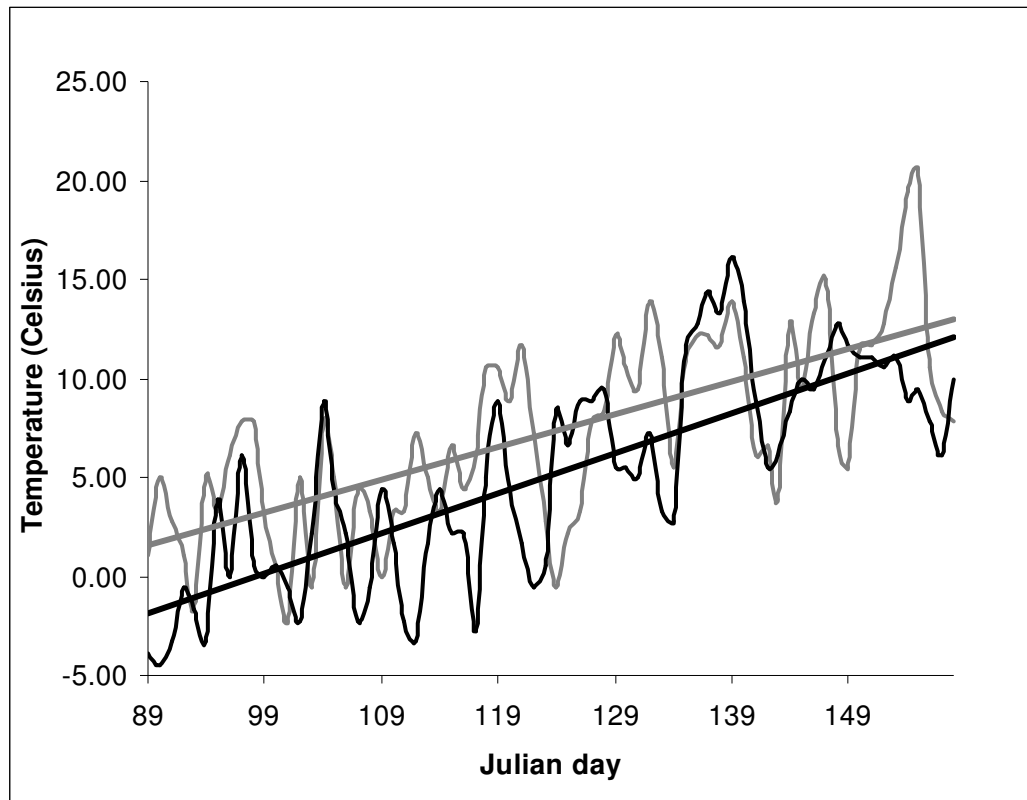Turner 1978, Murphy 1992). Timing of the germination of temperature-dependent

Figure 1.4. The daily minimum temperature (°C) in Boise, ID in 2007 (gray) and 2008

(black) from Julian day 89 to 158 during American kestrel incubation with trend lines.

plants would be different between field seasons, which could affect food availability (Chesson et al. 2004, Murphy 1992). The prey population could then influence maternal body condition as well as that of the offspring.

Androgens did not affect most variables concerning size and growth rate in my study, however many characteristics differed between years. Hatching mass was lowered with an increase of exogenous yolk androgens in the same year that exogenous androgens decreased survival, which suggests that androgens can be detrimental to development in some conditions.

Skeletal Growth

BALP was not affected by yolk androgens in this study, but measuring this alkaline phosphatase isoenzyme provides a unique perspective on growth. Morphological measurements provide information on the overall size of an organism, but they do not clarify the maturity of the structure measured. For example, tarsus measurements do not indicate the amount of ossification of the bone, only the general length. Longer legs reflect elongated bone growth, but that growth could be of poor quality; i.e., there could be little appositional growth, which is growth that strengthens the bone and helps prevent breakage. Looking at the levels of BALP in plasma can shed light on the effects of androgens on the quality of a nestling's skeletal structure because the amount of BALP found in plasma correlates to the amount of ossification occurring during development. Androgens may affect levels of BALP because they stimulate skeletal development (Vandenschueren et al. 1998).

The addition of yolk androgens had no observable effect on the amount of BALP in nestlings. The increase of androgens *in ovo* may not have lasted long enough to affect bone development on days 15 or 26, or the androgens may not have had any effect on BALP at all. Timing of the samples affects results. For example, domestic chickens (*Gallus domesticus*) show a low amount of ALP in the first few days after hatching, followed by a peak in the second week that is five times the level of an adult (McComb 1979). Perhaps taking blood samples closer to hatching would illuminate any initial differences in BALP caused by the addition of yolk androgens that disappear later in development, just as the initial effects of androgen treatment on hatching mass disappear.

Our understanding of the effect of androgens on developmental BALP levels in birds is limited. Nestlings are believed to have higher BALP levels because their bones are still in the process of ossification, unlike the bones of adults (Tilgar et al. 2004). BALP is higher during development, and small nestlings in a clutch show higher BALP levels than their larger siblings and may assist in "catching up" in size (Tilgar et al. 2004). However, in my study, the nestlings with lower hatching mass from the group given increased yolk androgens did not require elevated BALP to reach an equivalent body mass to the control nestlings by day 26.

An increase of BALP has been correlated to poor health in adult birds (Smits et al. 2007, McComb 1979). Thus, if the androgens affected some aspect of health or development, it may be reflected in the BALP levels. Most nestlings appeared healthy and were at least healthy enough to survive through fledging. Had there been any nestlings that survived through day 15 but did not make it to fledging, it would have been

interesting to compare their day 15 BALP levels to the nestlings that did survive to fledging, but all nestlings that survived to day 15 also survived to day 26.

Hematocrit

Hematocrit levels, the ratio of packed blood cells to the full blood sample, are correlated with oxygen transport. Specifically, higher hematocrit levels increase the amount of oxygen that can be transported, which supports higher metabolic rates (Saino et al. 1997). Therefore, an increase of hematocrit may allow birds to increase activity (Saino et al. 1997). In my study, exogenous androgens increased hematocrit levels in 2007, which corresponds to an increase of oxygen capacity, allowing greater metabolic rates for offspring with higher androgens (Saino et al. 1997). Clutches with the medium androgen dosage had higher hematocrit levels than the low dosage and control, similar to the human response to androgens. In humans, artificial increases of testosterone increase hematocrit levels (Simon et al. 2001), and the blocking of androgens causes a decrease in hematocrit levels sufficient to cause anemia (Qian et al. 2004). The increase in hematocrit in 15-day-old kestrel nestlings could represent an increase in metabolic activity, which, if present from hatching, could be responsible for the reduced hatch mass in birds from androgen-treated eggs discussed earlier. An increase in hematocrit may also result from dehydration (Vleck and Priedkalns 1985), and this, too, could lower the hatch mass. In 2008 there was no treatment effect on hematocrit. Consistent with my 2008 findings, implants of testosterone in yellow-legged gulls (*Larus cachinnans*) did not change gull hematocrit levels, but the birds were captive and adults unlike this study (Alonso-Alvarez et al. 2002). More research examining hematocrit after altering

androgens needs to be done to get a clearer picture of the effects of androgens on avian hematocrit.

American kestrel hematocrit levels can vary seasonally and are affected by environmental conditions (Fernie and Bird 2001). However, separating external effects from the effects caused by androgens is difficult. Julian day was a significant effect for hematocrit levels. When variation from Julian day was pulled out as a covariate, the effect of androgens remained. The significant effect of Julian day on hematocrit level was most likely influenced by the species' circadian rhythms (Ferrer 1990, Rehder and Bird 1983). Hunter and Powers (1980) found that hematocrit levels change with season for many raptors in Idaho. In that study, adult American kestrels showed the lowest levels of hematocrit in the breeding season between March 20th and April 14th in 1976.

My data did not exhibit year effects, but results did differ between years. Day 15 hematocrit levels in 2007 were significantly affected by androgens, yet hematocrit levels in 2008 were not affected. Differences possibly resulted from environmental differences between the two years. Temperature may have influenced this difference as Rehder et al. (1982) found an inverse relationship between hematocrit and temperature in kestrels. In another multi-year field study, Potti et al. (1999) did not find year differences for hematocrit in pied flycatchers (*Ficedula hypoleuca*), but environmental variability between the years was not mentioned. A relationship may exist between androgens, hematocrit, and the external environment, but their potential effects and interactions are not well understood.

<u>Secondary Sexual Characteristics</u>

Androgens can enhance sexually selected plumage characteristics in adult males (Strasser and Schwabl 2004, Peters et al. 2000). In my study however, subterminal tail bands, a secondary sexual characteristic known to affect mate choice in kestrels (Weihn 1997), did not differ significantly among treatment groups. Although this result may indicate that androgens have no effect on subterminal tail bands, at least within the range of dosages included in my study, it is possible that low sample size may have resulted in a type II error - males were present in only 16 of 44 clutches in 2007 and 2008 combined. Thus, a larger sample size may be required before any conclusions can be made about the effect of androgens on the subterminal tail bands of male kestrels.

The left and right tarsi and wing chords did not show any difference in fluctuating asymmetry among treatments. Though Quinn et al. (2005) suppressed androgens, they found no difference in fluctuating asymmetry after manipulation either. Furthermore, androgens did not affect the symmetry along the right and left sides of the kestrels in my study, and therefore are unlikely to have affected mating preference (Møller and Thornhill 1998).

<u>Immunoresponsiveness and Heterophil to Lymphocyte Ratio</u>

Whereas many studies have found that androgens lower immunoresponsiveness (Groothuis et al. 2005a), my study found no effect of exogenous yolk androgens on immune responses. No significant effect of PHA treatment or H/L ratios occurred in the various experimental groups. Androgen levels are related to cell-mediated immunity, but perhaps not directly. For instance, cell-mediated immunity decreases after long periods

of T elevation, but this may not be from the direct influence of T, but the effect of T on corticosterone (Casto et al. 2001, Navara and Mendonça 2008). Elevated yolk androgens in American kestrels increase levels of corticosterone, which has a negative correlation to body condition (Sockman and Schwabl 2001). The effects of corticosterone on immunocompetence may be indirectly caused by androgens; however, it was not observed here.

Both measurements with PHA and H/L ratios did not find a significant difference of an effect of androgens. Tschirren et al. (2005) did not find that androgens affected PHA in great tits. Navara et al. (2005) found less swelling from PHA using multiple dosage levels of testosterone, but found no effect on the H/L ratio. Similarly, while manipulating brood size created differences with PHA and testosterone, no differences in H/L ratios were found in songbirds (Naguib et al. 2004). While many studies find that androgens decrease immunoresponsiveness (Quinn and Ottinger 2006), the contrasting results in studies such as those by Tschirren et al. (2005) and Naguib et al. (2004) are producing a more complex picture of how yolk androgens can affect the immune system during development.

Since H/L ratios correlate to body condition and androgens did not influence the ratio of these leukocytes, exogenous yolk androgens may not have affected body condition on days 15 and 26. This is similar to the lack of difference found with the PHA measurements taken on day 26 in 2007. While PHA is more specific to immunosuppression, both were used to look at effects on white blood cells. Androgens may not affect the ratio of different types of white blood cells or the amount produced. The lack of difference among the treatments is consistent with the results measured on

day 26. If androgens did affect immunoresponsiveness, the effect did not last until day 26. External factors may have clouded results by that point in time as well. I suggest that PHA injections should be done at a younger age to see if androgens affect cell-mediated immunity, even if it does not last till fledging.

Maternal testosterone has an indirect relationship with antioxidants in eggs (Royle et al. 2001). Antioxidants may boost immune system support, and their absence would increase the effects of free radicals, which could harm body condition. How androgens function with the immune system and their interactions with chemicals associated are still not known. Injecting only androgens into eggs without providing other substances that might interact with androgens in biochemical pathways may result in the absence of developmental effects (Sockman et al. 2006). It is critical to understand the interactions between androgens and other material. My experiment increased only yolk androgens and did not change the amount of antioxidants (or other biochemicals) the mother had already deposited in the egg, which may have contributed to the absence of a change in immune response.

H/L levels increased significantly from 2007 to 2008. However, T cell-mediated immunity was not measured in both field seasons, and it would be interesting to see if the swelling from the PHA injections also varied between years since environmental effects such as food availability has been shown to affect T cell-mediated immunity (Martinéz-Padilla 2006). The year effect impacted the blood parameter analyzing immunity, which may assist in identifying the change that occurred between years.

<u>Effect of Year</u>

Variation from the lack of control in field experiments has both advantages and disadvantages. Experiments are more realistic in field settings, but external factors may influence patterns and results in ways that are unknown to the investigator or that cannot be controlled. Year affected tail length, wing chord, hatching mass, growth rate, and H/L ratios. Nestlings may react differently to the combination of androgens and the characteristics of each year like temperature, or, indirectly, androgens may be impacted by the different growth patterns of a nestling due to year effects. Females deposit differing amounts of androgens depending on her environment (Sasvári et al. 2004). The amount of androgens may not be necessary for development every year. Disregarding androgens, it is interesting to contemplate why certain nestling traits and not others were affected. The effect of different field seasons is important to acknowledge since it can expose the complexity of development in a varying environment.

From casual observations, the most obvious environmental differences between the two years were from temperature and precipitation. Differing temperature and food availability influencing parental condition between years or habitats can affect egg composition (Bourgault et al. 2007, Hargitai et al. 2006). It is possible that weather conditions modified the amount/effect of yolk androgens in the eggs through parental condition (Sasvári et al. 2004). The unknown year effect may have influenced habitats differently causing the 2007 higher nest success for the Boise clutches in more natural environments compared to the Meridian boxes in more suburban and agriculture areas. Not only mean temperature, but also the variability of temperature can affect egg composition and embryo development (Pendlebury and Bryant 2005). Low temperature

can slow embryonic development, but the potentially harming effect of temperature during development may be mediated by the effect of yolk androgens (Martin and Schwabl 2008).

Overall, winter and early spring precipitation amounts were similar in 2007 and 2008, but differed in monthly pulses. Pulsed resource availability in semi-arid regions forces organisms to take full advantage of resources while they exist, shifting the timing of growth (Chesson et al. 2004). Prey availability may have differed temporally between seasons and may have affected parental condition directly before egg laying though laying dates did not differ between years (Bourgault et al. 2007).

Temperature and precipitation may have contributed to the divergent survival rate results and the effect of year for tail length, wing chord, hatching mass, growth rate, and H/L ratios. However, since my study did not focus on annual variation in environmental factors, the causative factors underlying the year effects shown in my data remain an open question.

Phenotypic Plasticity

Androgens have been suggested to have costs (such as lowered immunity) and benefits (increased strength and size) (Groothuis et al. 2005b), but neither was detected in either year of my study. Androgens have distinct roles in the development of nestlings; however, the variation in androgens may be a side product of the egg laying process. Ideally, the female would produce offspring with the phenotype that would enhance its chances at survival. In American kestrels, females lay eggs with increasing levels of yolk androgens. Yolk androgens have been shown to affect a variety of morphological and

physiological parameters, all of which suggests that yolk androgens are involved in generating phenotypic plasticity. That range of phenotypes would not only be dependent on the yolk androgens, but also the surroundings. The variation in androgen levels from the first egg to the last egg may result in a wide spectrum of physiological results, each of which could be ideal in different environmental conditions. Females can adjust egg-laying behavior with their environment to produce offspring with phenotypes that give the optimal chance at survival (Badyaev and Oh 2008). When clutches are smaller later in the season, American kestrel females can increase the amounts of androgens more rapidly, so the fourth laid egg of a small clutch would have the same amount of androgens as the sixth egg in a larger clutch earlier in the season (Sockman et al. 2006). Here, different dosage levels did influence the survival rate, so with natural androgen variation in eggs, some eggs may be more likely to hatch and fledge than others. The odd aspect of my results is both the clutches given the lowest androgens (control) and the highest androgens had the highest survival rate. The success of the clutches with variable offspring may not result in the highest overall success, but variable offspring may results in more consistent success from year to year (Gillespie 1977, Laaksonen 2004).

Another explanation for the variable steroid allocation, the hatching asynchrony adjustment hypothesis, suggests that steroid levels are adjusted to assist in the development of the last laid eggs, allowing them to catch up to the first hatched nestlings (Groothuis et al. 2005a; but see Schwabl 1996). However, the American kestrel nestlings with the medium dose of androgens had a lower hatching mass in 2007, yet those nestlings matched the other treatment nestlings by day 26. They did catch up in size, but potentially at the expense of quality maturation of the tissues (Ricklefs 1979). The

increased hematocrit from androgens may give a metabolic boost, but those results also occurred only in one year. The hatching asynchrony adjustment hypothesis was not supported here, but a clearer image may be discerned if hatching order was determined to have specific effects on eggs laid later in sequence.

Possible tests to elucidate the influence of androgens include pulling out yolk samples to test androgen levels in the field and concurrently observing the success level of the nests. Nestlings with certain natural levels of androgens may have a higher survival rate than other nestlings, as I found was the case with exogenous androgens. Repetitions over multiple years with different environmental conditions can determine if androgens have differential effects on nestlings. In general, most androgen studies are short term, so differences due to yearly effects may be incorporated as overall results. With a long-term study, a pattern of success could be found among nests with different androgens levels or with low overall variation and high variation. If the breeding environment is the most important unpredictable factor, then American kestrel clutches in more stable environments should have lower variation of androgens within a clutches than geographical areas with high environmental fluctuations. Lab tests so far have not tested the effects of androgens of a population in different environments over multiple years, and clutches incubated in different temperatures may show varying consequences of androgens.

Conclusion

Yolk androgens are anabolic steroid contributions given by a mother to her offspring that influence the offspring's phenotype. Over the last decade, research on the effects of yolk

androgens has found a myriad of results. One was of increasing growth and strength, but

Sockman and Schwabl (2000) found a different impression with American kestrels where

androgens lowered their survival and mass. My study found a mix of both pictures.

Androgens lowered hatching mass and increased hematocrit levels, but these effects were

not apparent by the time of fledging. Thus, androgens do affect development, but other

factors may have overcome yolk androgen effects by fledging. The effects found in the

2007 field season were not apparent in the 2008 field season, so the effects of androgens

are linked to the surrounding environment somehow. Finally, the level of androgens used

by Sockman and Schwabl (2000), as well as the control level, both had the highest

number of nestlings that hatched and survived to fledging; smaller androgen dosages had

less nestlings hatch and fledge. Therefore, the amount of androgens in the yolk can affect

offspring. The female may manipulate yolk androgens as a tool to alter offspring

phenotype, but interactions with the environment could create varying yolk androgen

effects that require additional research to understand.

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CHAPTER 2: GETTING A GRIP ON MEASURING

KESTREL DIGITS

Introduction

The link between digit ratios and androgens is a popular topic in the research community,

but the digits can be difficult to measure. In humans, the ratio between the second and

fourth digit (2D:4D) has been used as a sign of prenatal androgen influence that is

associated with future behaviors and diseases (McIntyre 2006). In birds, digit ratios are

linked to androgen deposition in the egg (Burley and Foster 2004). Zebra finch

(*Taeniopygia guttata castanotis*) females with less androgens in the eggs, as determined

by higher digit ratios, are more likely to prefer to mate with attractive males, suggesting

that androgens may affect sexual selection (Burley and Foster 2004). I attempted to

measure digit ratios in American kestrel nestlings, but my methods produced erratic

results that would be inappropriate to rely on. Nonetheless, analyzing the effects of yolk

androgens on digit ratios may lead to a better understanding of development and the

effects of yolk androgens, so the development of a suitable technique to measure digit

length would be valuable.

Studies of digit ratios of different animal species are inconsistent in the digits

compared and the appendage used (McIntyre 2006, Burley and Foster 2004). In the ring-

necked pheasant (*Phasianus colchicus*) the 2D:3D ratio was compared, but effects from

androgens were found only in the females (Romano et al. 2005). Burley and Foster (2004) looked at the 2D:4D ratio of the zebra finch and found it increased with a natural decrease in circulating androgens, but the ratio also differed between the sexes. The 2D:4D digit ratio was compared in adult collared flycatchers, *Ficedula albicollis*, to see if it relates to testosterone and other characteristics associated with yolk androgens (Garamszegi et al. 2007), but the relationship between digit ratio and maternal androgens was unclear. With no clear pattern to the results, it is critical that the methodology of digit measuring be consistent and repeatable to ensure that technical issues do not contribute additional variability to the data. In my study, I used what turned out to be an unreliable technique, but an improved technique for the measuring digits of the strong-footed American kestrel could allow investigators to determine the effects of yolk androgens have on kestrel development.

Methods

To measure digit asymmetry during the 2007 and 2008 field seasons I pressed the right and left feet of the American kestrel nestlings into flattened piece of clay about 0.5 cm thick set on a smooth surface. Prints were discarded if the kestrel did not relax the tension in the foot; that is, if the bird gripped the clay as opposed to "stepping" into the clay. Each digit was measured from the top of the middle footpad to the tip of the talon, and this measurement was compared to the corresponding digit on the other foot.

Results

Many data points were discarded because of variation in digit tension, and digit measurements were still not consistent. I do not believe that they were accurate enough to be interpreted with statistical testing. See Table 2.1 for means and standard deviations.

Discussion

The method of holding the foot open and making an imprint in clay must be improved if it is to be used in raptors to measure the level of asymmetry between the left and right digits. Measuring the digit length was very erratic and inconsistent because the nestlings would sometimes have relaxed feet, creating relatively long digit length measurements, and sometimes clenched feet, yielding relatively short digit length measurements. Also, occasionally one foot was clenched and the other was loose, making the measurements even more untenable. Each age range had its difficulties: when prints were taken from birds between day 5 and 10 days of age, the prints in the clay had varying levels of tension and looseness. Right before fledging, the nestlings would either have a perfect splay of the digits or be extremely tense. If using clay to measure the digits, I recommend, with both age groups, to place the clay on a flat surface, hold the bird with a bander's grip (the head between the "V" made by the middle finger and pointer finger and the rest of the fingers wrapped around the sides and resting gently but securely around the breast and stomach), and try to extend the nestling's legs as far out as possible and have them "stand" on the clay ready to lightly press the tops of the feet into the clay (see Figure 2.1). The impression does not have to be deep, but pressed hard enough to make a visible print.

Table 2.1. Absolute differences (in mm ± the standard deviation) between the left and right digit for the second, third, and fourth digits in 2007 (N= 25) and 2008 (N = 19). Day refers to the number of days after hatching.

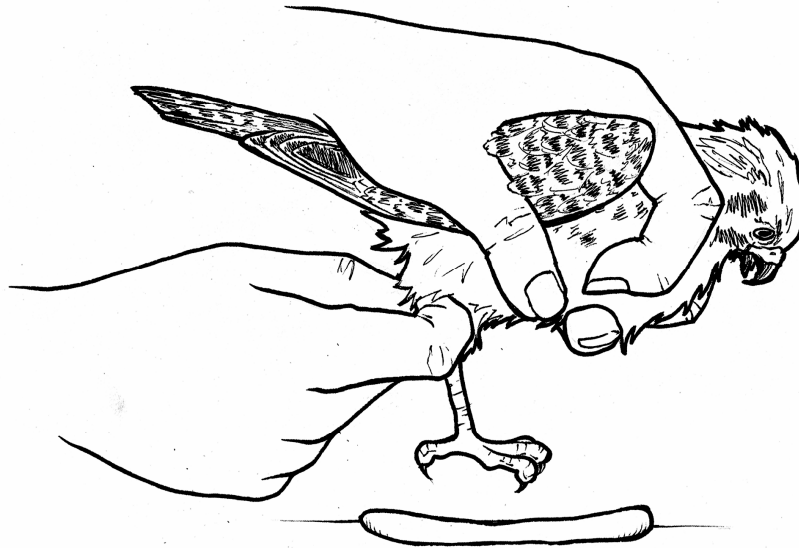| Year	Day	Digit	Treatment			
			Control	Low	Medium	High
2007	10	2	1.43 ± 1.38	1.71 ± 1.92	1.55 ± 1.41	
		3	1.45 ± 0.87	1.01 ± 0.53	1.58 ± 1.28	
		4	1.67± 1.52	1.04 ± 1.01	2.17 ± 1.81	
	26	2	1.67 ± 1.61	1.60 ± 1.42	1.64 ± 1.20	
		3	1.83 ± 1.34	2.06 ± 1.35	2.05 ± 1.86	
		4	1.26 ± 1.38	1.57 ± 2.43	1.97 ± 1.93	
2008	5	2	1.09 ± 0.50		1.45 ± 0.93	1.45 ± 1.02
		3	1.36 ± 1.08		1.50 ± 1.92	2.25 ± 1.26
		4	0.87 ± 0.49		0.99 ± 0.57	1.43 ± 1.02
	26	2	1.76 ± 1.50		1.26 ± 1.19	1.87 ± 2.02
		3	2.51 ± 1.07		2.08 ± 1.65	2.02 ± 1.75
		4	2.04 ± 1.68		1.11 ± 0.89	1.87 ± 1.30

Figure 2.1. Handling procedure to increase accuracy of digit measurements using clay. The hand not restraining the bird will pull the leg out to loosen the digits. To get a stronger imprint, the forefinger may be slid down the foot to lightly press the digits into the clay.

Another possible method would be to use a dowel with millimeter hatch marks around the circumference that the nestling could perch on. Using a smooth dowel that the nestling could grip comfortably, the beginning and end of the digit could be marked (see Figure 2.2). Then the measurement could be taken after the bird is released. Different sized dowels may be used for different ages within the same species.

Another potential issue concerns the continuity of measurements; where to begin and end the digit measurements can vary depending on the observer. I would recommend measuring from the tip of the digit pad to the crease between the footpad and digit. I completed all of the measurements in this study, but if multiple observers took measurements, then it would be very easy to increase variation.

With creativity and persistence, a simple and accurate method of digit measurement may be created for American kestrels that will also be useful for other avian species. An increase in precision will create an increase in quality results.

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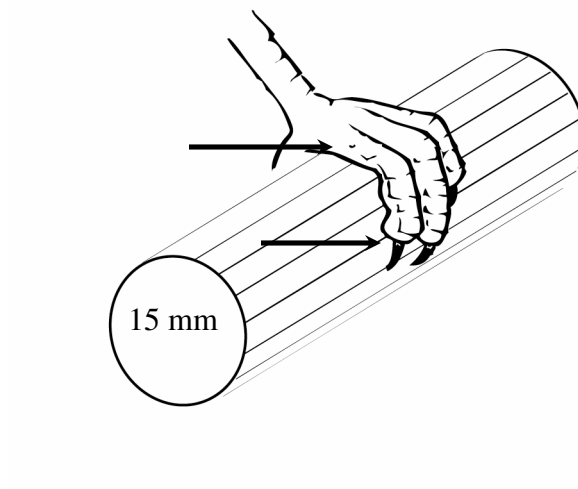
Figure 2.2. Dowel technique to measure digit length. Marks can be made at the beginning of the digit and at the end.