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Yolk androstenedione in domestic chicks (*Gallus gallus domesticus*): Uptake and sex-dependent alteration of growth and behavior

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ABSTRACT

In birds, causes and consequences of variation in maternally-derived steroids in egg yolk have been the subject of intense experimentation. Many studies have quantified or manipulated testosterone ("T") and one of its immediate precursors, androstenedione ("A4") – often lumping the two steroids as "androgens" and treating them as functionally equivalent. However, yolk A4 is deposited in substantially higher concentrations than T, binds only weakly to the androgen receptor, and is readily converted into either T or estrone by steroidogenic enzymes present during embryonic development. Thus it may not be appropriate to assume that A4 has the same effect as T. In addition, A4's metabolic fate is likely to differ between females and males. The goals of this study were to examine the sex-specific uptake and metabolism of yolk A4 and consequences of elevated levels of yolk A4 on development and behavior of domestic chicks. Eggs were injected with 2μ Ci of tritiated androstenedione; radioactivity was detected in all tissues of day 7 and day 16 embryos and found in both aqueous and organics phases of day 7 yolk, with no difference between sexes. A second set of eggs was injected with 125 ng of A4. A4 increased growth of morphological traits (tarsus, beak) in females, but not males. A4 males had smaller combs than controls; there was no treatment effect in females. A4 reduced tonic immobility behavior in both sexes. The results of this study illustrate the importance of distinguishing both between androgens and between sexes when investigating avian endocrine maternal effects.

1. Introduction

The chemical environment for developing animals represents the first "environmental" contribution to phenotype, and in oviparous animals is determined entirely by mothers during egg formation. In birds the contents of eggs can vary dramatically both among and within species, and in response to a number of developmental, physiological and environmental factors (Gil et al., 2007; Groothuis et al., 2005; Schwabl, 1993); this variation can have significant consequences for chick phenotype (reviewed in Navara and Mendonça, 2008). There is a particularly large body of research documenting causes and consequences of variation in yolk steroids, with a heavy emphasis on yolk androgens, specifically testosterone ("T"; reviewed in Navara and Mendonça, 2008). However, there is mounting evidence that levels of the androgen androstenedione ("A4") in eggs may be an important maternal effect that varies in response to different factors than T (Gil et al., 2007; Groothuis and Schwabl, 2002; Tschirren et al., 2009) and may induce different phenotypic changes (Hegyi and Schwabl, 2010).

Androstenedione is a weak androgen with a low affinity for the androgen receptor (Holterhus et al., 2002), (reviewed in Groothuis and Schwabl, 2008). It is present in large quantities in yolk – usually at levels many times higher than T (reviewed in Gil et al., 2007) – and appears to be actively taken up from yolk by developing avian embryos (Eising et al., 2003; Elf and Fivizzani, 2002). A4 is often assumed to be a T precursor and frequently lumped with T (and sometimes dihydrotestosterone) as "androgens", both for quantification (Gasparini et al., 2007) and manipulation (Eising and Groothuis, 2003; Müller et al., 2012; Pitala et al., 2009; Saino et al., 2006a; Sockman et al., 2007) purposes. However, it is becoming clear that the causes and consequences of variation in yolk A4 are different from those of yolk T (Gil et al., 2007; Groothuis and Schwabl, 2002; Hegyi et al., 2011; Hegyi and Schwabl, 2010; Tschirren et al., 2009). Despite emerging awareness about yolk A4, we still do not know enough about the effects of exposure to elevated A4 on phenotype to establish clear hypotheses about how maternal deposition of A4 may have been shaped by selection (Groothuis and Engelhardt, 2005). To our knowledge, thus far only one study has experimentally manipulated yolk A4 without concurrently manipulating T (Hegyi and Schwabl, 2010). That study found that A4 had different effects on quail chicks than T. While that study was not able to distinguish between the sexes, in many species male and female chicks respond differently to maternal

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steroids (Ruuskanen et al., 2012; Tobler and Sandell, 2009). Thus understanding the potential role of yolk A4 may require determination of the sex-specific consequences of exposure.

In this study, we assessed tissue-specific distribution of radio labeled yolk A4 in male and female embryos at two ages (day 7 and day 16 of embryonic development; hatching occurs on day 21) and phase separation (organic v. water soluble) of radio labeled A4 derivatives in day 7 yolk to test the hypothesis that yolk A4 is taken up and metabolized differently by male and female embryos. Day 7 was chosen because it is an age at which gonads have just begun differentiating, and steroidogenic enzymes are being expressed in a sex-dependent way (Bruggeman et al., 2002). Day 16 is well beyond the termination of sexual differentiation (day 11; Bruggeman et al., 2002), and within a window of time in which yolk estradiol levels surge (Elf and Fivizzani, 2002), suggesting a dynamic time for steroid metabolism and steroid exchange between yolk and embryo. We subsequently assessed the effects of exposure to elevated yolk A4 *in ovo* on (a) morphology of hatchling chickens, (b) post-hatch growth, and (c) tonic immobility behavior to test the hypothesis that yolk A4 has different effects on the phenotypes of developing male and female birds. We predicted that developmental exposure to elevated A4 would have sex-dependent effects on phenotype that differed from those resulting from exposure to elevated T, though comparisons across hormones and species must be made tentatively.

2. Methods

2.1. Experiment 1: embryonic uptake of yolk A4

2.1.1. Egg treatment and tissue collection

To assess the uptake and tissue-specific distribution of yolk A4, we injected eggs with tritiated A4. Fertilized unincubated Bovan White chicken eggs were obtained from Centurion Poultry, Inc (Montandon, PA). 25G butterfly needle infusion sets were used to inject 37 eggs with 50 μl sesame oil containing 2 μCi of tritiated A4 (1β, 2β-3H; American Radiolabeled Chemicals, Inc. Art 0648). All eggs were incubated at 37.5 °C in a circulated air model Hova-Bator™ incubator and automatically turned once per hour. On day 7, 12 eggs were collected and divided into embryo head, body, albumin and yolk/yolk sac (embryos were small enough that separating organs was not feasible). On day 16, 14 eggs were opened, blood was collected from a blood vessel on the outer surface of the yolk sac when possible, embryos were euthanized and the brain, heart, a liver sample, a pectoral muscle sample, and a combined sample of extraembryonic fluids and membranes (yolk sac, allantois and membranes – albumin was no longer apparent) were collected. Yolk, albumin and extraembryonic fluids were homogenized with water via sonication and vortexing with glass beads, while all other tissues were diluted with water, ground with a pestle and vortexed. Aliquots of samples were then counted in duplicate on a scintillation counter.

2.1.2. Separation of aqueous and organic phases

To assess sex-dependent conversion of yolk A4 into polar metabolites, ~500 μL of yolk from day 7 eggs (6 males, 4 females) was separated into aqueous and organic components using a double extraction with 3 mls of 70:30 diethyl ether:petroleum ether, following the protocol described by Paitz et al. (2010). Radioactivity of each phase was quantified using a scintillation counter.

2.1.3. Sexing

Tissue samples were used to molecularly sex all embryos following the PCR and gel electrophoresis protocol of (Fridolfsson and Ellegren, 1999).

2.2. Experiment 2: effects of yolk A4

2.2.1. Egg and injection

In a separate experiment, fertile, unincubated eggs from the same source were weighed, numbered and assigned treatments such that distribution of egg masses was the same in two treatments. 101 of 120 eggs weighed between 60 and 70 g and were therefore included in the study. "A4" eggs were injected with 125 ng of A4 dissolved in 50 μL of expeller pressed sesame oil; "C" eggs were injected with 50 μL of expeller pressed oil. Fresh yolks of the eggs from our supplier averaged 16 g, thus the injection was equivalent to approximately 7.8 ng A4/g yolk. This dose was based on yolk A4 levels reported in unincubated white leghorn chicken eggs, for which one study found that eggs contained an average of 35 ng/g yolk with a standard deviation of approximately 23 ng/g (Müller et al., 2002), and another study found that levels varied from 10 ng/g to 36 ng/g with a standard deviation of approximately 13 ng/g (Elf and Fivizzani, 2002). Thus the dose used here is probably conservative in elevating yolk A4 less than one standard deviation above mean values.

Injection solutions were made by dissolving powdered 4-androsten-3,17-dione (Steraloids, Inc.) in ethanol, adding 125 μL of the ethanol solution to 5 mLs of sesame oil, and conducting multiple rounds of vortexing and incubation in a 37 °C water bath until ethanol was evaporated. On each egg, a site approximately 10 mm below the intersection of the longitudinal and latitudinal center lines was prepped with a 70% alcohol swab. A hole was manually drilled through the shell using a small drill bit. A 100 μL Hamilton syringe with a luer tip and a 25G 1" inch disposable needle (BD #305125) was fully inserted at a 45 degree angle towards the airsac at the blunt end of the egg (a distance that places the needle tip in the middle of the yolk), and 50 μL was gently ejected. Holes were sealed with a dot of superglue, eggs allowed to rest at room temperature for at least 5 h, then placed in an incubator. The effectiveness of this technique was validated by injecting red food coloring into 20 practice eggs in the same manner, and verifying that the dye was found solely in the yolk.

2.2.2. Egg & chick husbandry

Eggs were incubated for 19 days in a Brinsea OVA-easy incubator set at 37.5 °C and 55% humidity (regulated with an automatic pump) and automatic rotation every 90 min. Three days prior to hatch, eggs were transferred to wire hatching trays with individual partitions and placed in a GQF Digital Hatcher, set at 36.6 °C and 60–70% humidity with no egg rotation. On day 21 of incubation, the hatcher was checked twice/day for hatchlings.

Hatchlings were allowed to dry in the hatcher, then weighed and banded with a unique wing band. Bovan white chickens can be sexed by the pattern of primary feathers, so hatchlings were sexed, had tarsus measured and were placed in a brooder (Brower Model CQB20) set at 35 °C with access to *ad libitum* food (Purina Mills Start and Grow, Sunfresh) and water. Brooder temperature was reduced approximately 3 °C/week. Photoperiod in the room was 12:12LD. Chicks were weighed in the mornings within 1 h of lights-on in order to obtain post-absorptive mass. Chicks were euthanized after 26 days, and sex verified by gonadal morphology. The following final morphological measurements were taken postmortem: tarsus (diagonal), wing (flattened wing chord), beak (culmen; upper mandible from start of feathering to tip) and comb size (estimated using a size index, which was the product of comb length and comb height). Each measurement was repeated 3 times and the average used for analyses.

2.2.3. Behavior: Tonic immobility

Tonic immobility tests were conducted as described in Niall Daisley et al. (2005). On day 3 post-hatch, chicks were taken

individually from the brooder to a nearby room, placed carefully on their sides, and gently held down. If a chick did not release itself from immobility (begin to right itself) after 15 s, it was finished testing, assigned a score of "1", and returned to its brooder. If it did right itself, the test was repeated up to 6 times, stopping at the first trial in which the chick remained immobilized. A chick's tonic immobility score ("TI score") was the trial number at which it first remained immobile for 15 s, with a conservative score of 7 assigned to the 5 chicks that were not immobilized after 6 trials (1 C female, 2 A4 males, 2 A4 females).

2.3. Statistical analysis

2.3.1. Yolk androstendione uptake

Because we were not able to cleanly separate and weigh the entirety of most tissues, we were only able to establish whether A4 or its derivative(s) was present in each tissue, as indicated by the presence of radioactivity, and whether its concentration differed between the sexes for each particular tissue. Thus for each age and tissue we ran a *t*-test to determine effects of sex on concentration of radioactivity/g tissue. Effect of sex on proportions of radioactivity found in the aqueous and organic phases of day 7 yolk was tested using ANOVA with sex, phase and sex by phase interaction terms as factors.

2.3.2. Effects of yolk A4

All data met the assumptions of parametric testing, with the exception of the tonic immobility scores. For tarsus and body mass, measurements collected at hatch and at 26 days post-hatch were used in two separate repeated-measures ANOVAs (one for each sex) with treatment as the independent variable. For measures that were only taken at the end of the experiment (wing length, beak length and comb size), the effects of sex, treatment and their interaction were included as factors in an ANOVA. The proportion of chicks in each treatment that were rendered immobile on the first trial was assessed using a chi-squared analysis. Tonic immobility score data were not normally distributed and were therefore tested non-parametrically. Because non-parametric testing does not permit interaction terms, the effect of treatment was tested in separate analyses for each sex using a Kruskal–Wallis test.

2.4. Experiment 1: uptake of yolk A4

Fifteen of the 19 eggs collected at day 7 contained visible embryos (12 were analyzed), as did 14 of 19 collected on day 16. Due to logistical oversights, not all day 7 albumin samples were weighed, thus concentration of radiolabel in albumen could only be calculated for 2 of the females. Tritiated A4 or its metabolites (it was not possible to distinguish with these data) were found in all tissues examined at both stages of development, but there were no differences between males and females (p >0.05 for all ages and tissues; Fig. 2). Some tissues were not sampled from some individuals, generating variation in sample sizes (sample sizes reported in Fig. 2). Radioactivity was not detectable in yolk or albumin of uninjected eggs.

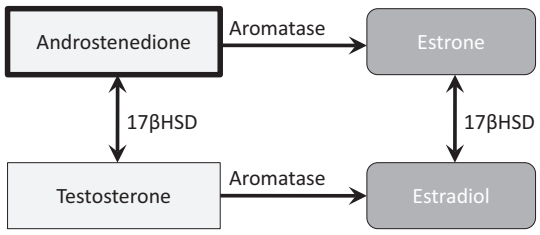

Fig. 1. Simplified schematic of androgen metabolism (modified from Kanehisa et al., 2011). Steroids are indicated in boxes, arrows indicate enzyme-catalyzed reactions, with arrows indicating directionality of reaction. Not all potential fates for steroids are indicated.

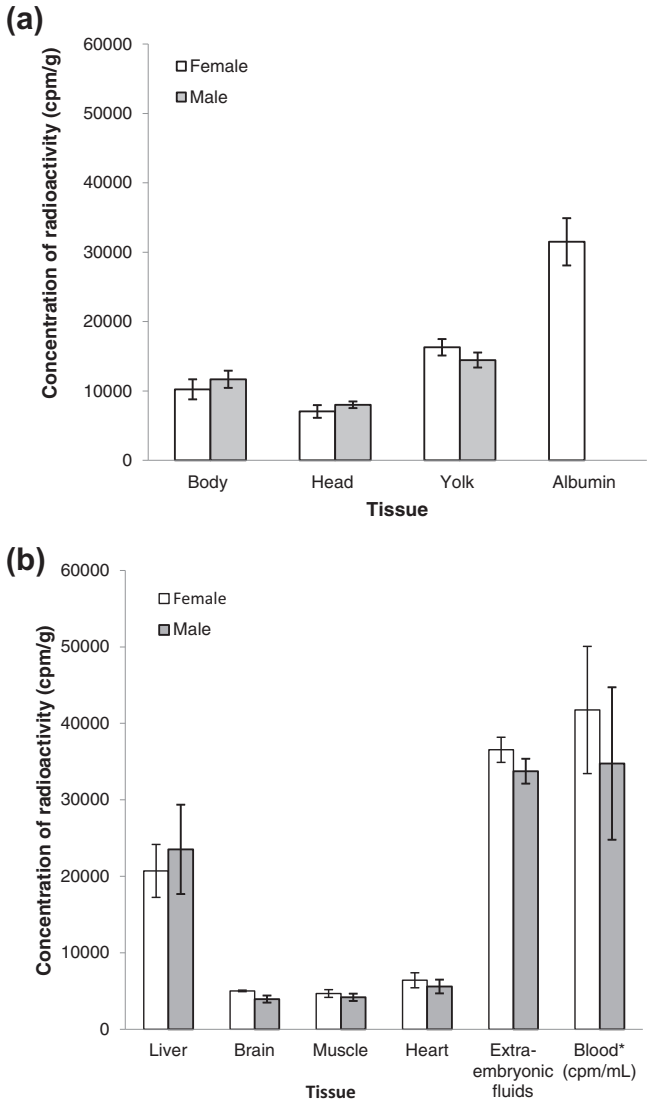

Fig. 2. Concentration of radioactivity in tissue samples collected on (a) day 7 of embryonic development (n = 6 for all groups except albumin (n = 2)) and (b) day 16 of embryonic development. Day 16 sample sizes (male, female): liver (n = 6, 5), brain (n = 8, 6), muscle (n = 7, 6), heart (n = 8, 6), yolk (n = 6, 6), blood (n = 4, 4). Radioactivity was present in all tissues sampled, and did not differ between the sexes.

Significant radioactivity was detected in both organic and aqueous phases of day 7 yolk (Fig. 3). A larger proportion of radioactivity was found in the organic than aqueous phase for both sexes (effect of phase: $F_{1,16}$ = 48.1, p <0.0001), and though there was a significant phase by sex interaction (effect of phase by sex: $F_{1,16}$ = 7.18, p = 0.016), this interaction was not reflected in post hoc tests, which only indicated the difference between phases, potentially due to the small sample size for females (n = 4).

2.5. Experiment 2: effects of yolk A4

2.5.1. Hatching success

Thirty-nine out of 50 (78%) A4 eggs hatched and 37 out of 51 (73%) control eggs hatched. Due to housing constraints, several chicks were euthanized at hatch – of these, three chicks from each

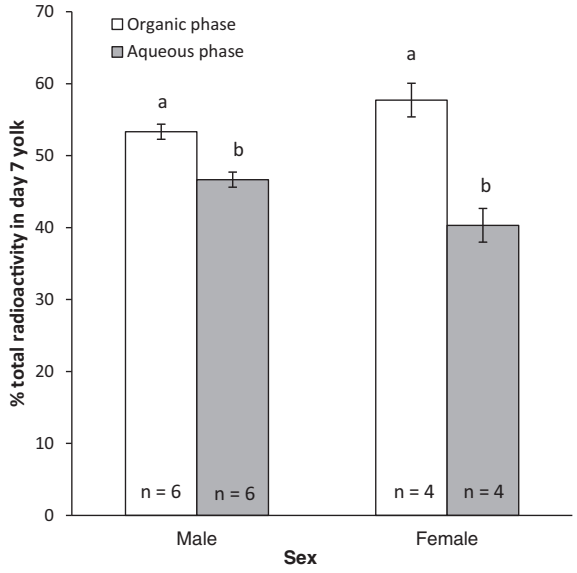

Fig. 3. Percent total detected radioactivity in aqueous and organic phases of yolk sac at day 7 of development in both males and females. Radioactivity was found in both phases, with a greater percent found in the organic layer and no differences between the sexes.

treatment were not sexed. Of the 68 chicks retained for the experiment, 22 of the males were from A4 eggs and 16 from control eggs; 13 of the females were from A4 eggs and 17 from control eggs. The number of males and females that hatched was not significantly different between treatments (p >0.05).

2.5.2. Growth & morphology

cBoth body mass and tarsus length were measured at hatch and at the end of the experiment (day 26 post hatch). Repeated measures ANOVA revealed no effect of treatment on males for either trait (Fig. 4; body mass treatment effect: $F_{1,31} = 0.43$, $p = 0.52$; tarsus treatment effect: $F_{1,32} = 0.118$, $p = 0.73$). In females, A4 had no effect on size of tarsus at hatch, but significantly increased growth of tarsus (day 26 tarsus was 44.09 mm ± 0.39 and 46.2 mm ± 0.43 for control and A4 respectively, Fig. 4; treatment effect: $F_{1,28} = 9.41$, $p = 0.0047$; age by treatment effect: $F_{1,28} = 10.75$, $p = 0.0028$), and showed a similar but non-significant trend for body mass (day 26 mass was 224.87 g ± 6.28 and 241.08 g ± 5.69 for control and A4 females respectively; Fig. 4; treatment effect: $F_{1,27} = 3.36$, $p = 0.07$; age by treatment effect: $F_{1,27} = 3.62$, $p = 0.067$).

After 26 days, there were no significant differences between treatments or sexes in wing length (Fig. 5; Table 1). Females from A4 treated eggs had longer beaks than those from control eggs (13.68 mm ± 0.15 and 14.13 mm ± 0.11 for control and A4, respectively), while there was no treatment effect in males (Fig. 5; Table 1). In contrast, a significant treatment by sex interaction effect reflected reduced comb size in males from A4 eggs, while females had smaller combs than males and showed no treatment effect (Fig. 5; Table 1).

2.5.3. Behavior

Both A4 females and A4 males were less likely to be immobilized on the first trial compared to control chicks (Fig. 6a; female: $\chi^2_{1,30} = 3.47$, $p = 0.008$; male: $\chi^2_{1,38} = 4.07$ $p = 0.043$). Female A4 chicks required significantly more trials to reach a state of tonic immobility than female controls (Fig. 3b; $Z = 2.64$, $p = 0.008$). Males

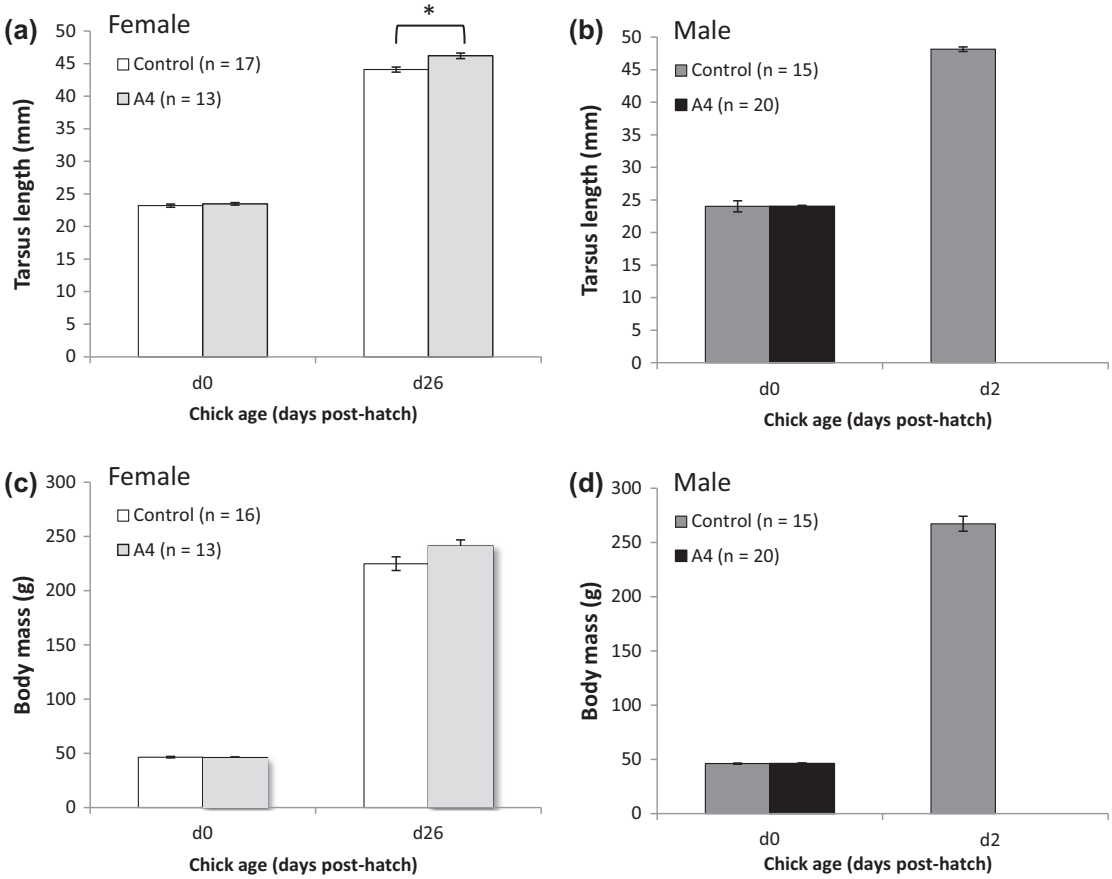

Fig. 4. Elevated yolk A4 did not alter tarsus length at hatch but increased it by day 26 post hatch compared to controls in females (a) and had no effect on male tarsus length at either age (b). Body mass showed a similar but non-significant response to treatment effect in females (c) and no response to treatment in males (d). Mean ± SEM.

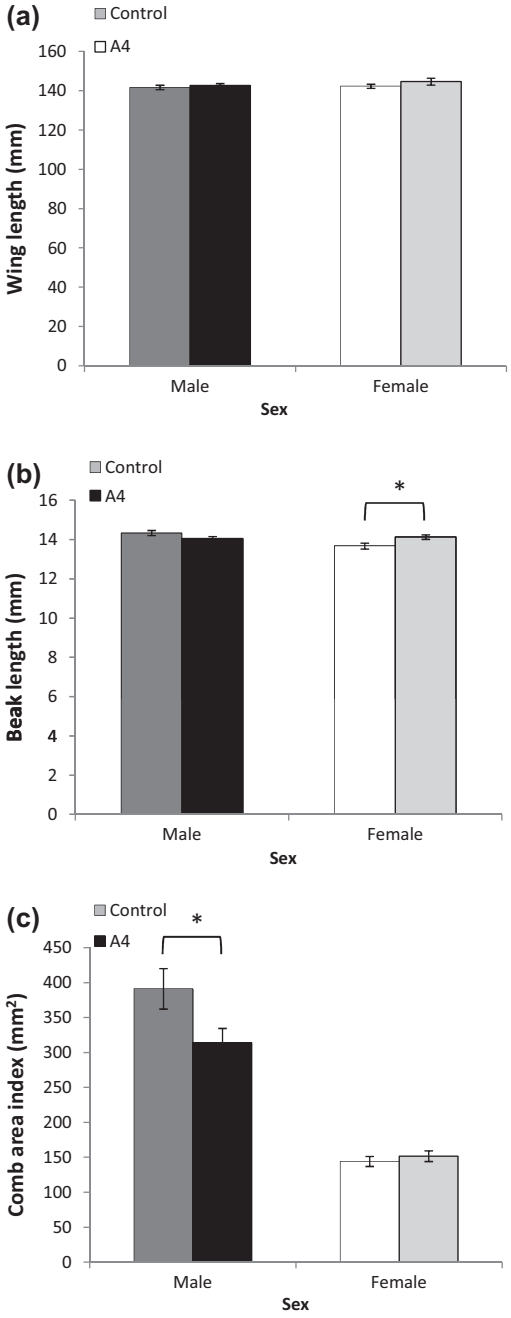

Fig. 5. At day 26 post hatch, there were no differences in wing length between treatments in either sex (a). A4 treatment increased beak size in females but not males (b), and reduced comb size in males but not females (c). Mean ± SEM.

Table 1
Effects of sex and treatment on morphological characters at euthanasia (ANOVA). Variation in degrees of freedom reflects missing measurements for some traits in some chicks. Bold values indicate $P < 0.05$.

	Wing	Beak	Comb
Overall model	$F_{3,63} = 0.97$ $P = 0.41$	$F_{3,61} = 4.64$ **$P = 0.006$**	$F_{3,61} = 38.37$ **$p < 0.0001$**
Treatment	$F_{1,63} = 1.97$ $P = 0.16$	$F_{1,61} = 0.38$ $P = 0.54$	$F_{1,61} = 3.21$ $P = 0.08$
Sex	$F_{1,63} = 1.11$ $P = 0.29$	$F_{1,61} = 5.00$ **$P = 0.029$**	$F_{1,61} = 109.82$ **$P < 0.0001$**
Sex * Treatment	$F_{1,63} = 0.32$ $P = 0.57$	$F_{1,61} = 8.32$ **$P = 0.005$**	$F_{1,61} = 4.75$ **$P = 0.03$**

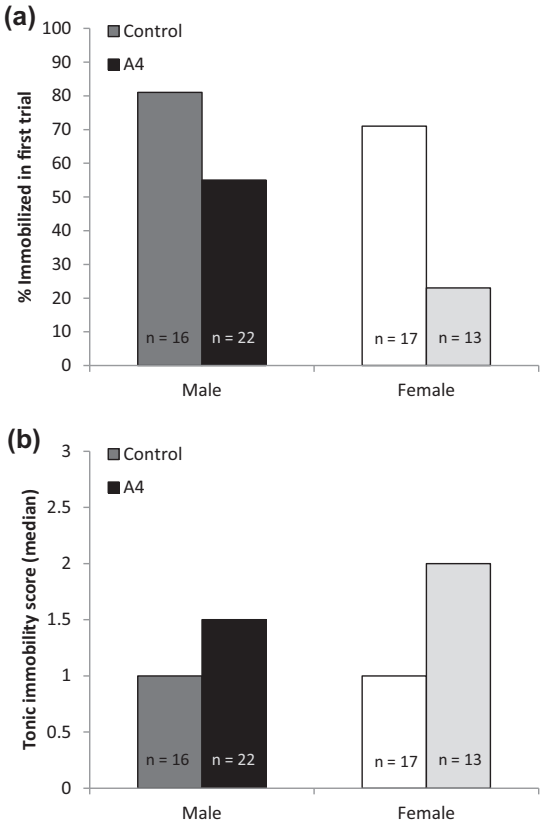

Fig. 6. (a) The proportion of chicks for which the first 15 s restraint trial induced tonic immobility for >15 s was significantly lower in the A4 treatment group for both sexes. (b) The median tonic immobility score was higher for A4 females than control males; males showed a trend for the same pattern. Tonic immobility score is the trial number at which chicks were successfully rendered immobile by a 15 s restraint.

showed a strong trend for the same pattern (Fig. 6b; $Z = -1.9$, $p = 0.056$).

3. Discussion

3.1. Summary

Yolk A4 is one of the most abundant steroids found in avian yolks, yet its metabolic fate, phenotypic consequences and mechanisms of action remain mostly unexplored. The data presented here support the hypotheses that, like other steroids found in yolk (Engelhardt et al., 2009; Paitz et al., 2010), A4 is taken up and metabolized by developing embryos (Figs. 2 and 3), and can generate sex-dependent changes in phenotype (Figs. 4–6). Growth of most traits was accelerated in female chicks from A4 eggs, but not affected in males, with the exception of comb size, which was inhibited in A4 males, but not females. The only other study to manipulate yolk A4 alone was conducted with Japanese quail, but the sex of chicks was not determined. They found that elevated A4 had similar effects on behavior as other androgens (dihydrotestosterone, T; promoting social reinstatement) but did not affect growth, unlike T which suppressed growth of body mass and tarsus (Hegyi and Schwabl, 2010).

3.2. Sex-specific effects of maternal androgens

Although the sex-specific consequences of hormone exposure are likely to depend on species-specific sexual dimorphism in growth and size as well as the selection pressures on that

dimorphism (Badyaev, 2002), there is a general (but not universal) pattern for female birds to be more sensitive to manipulation of the embryonic androgen environment than males. Several experiments that elevated yolk androgens (T or both T and A4) found that female growth was enhanced while male growth was either unaffected (Engelhardt et al., 2006) or compromised (Müller et al., 2008; Pitala et al., 2009). However, in some species the opposite is true, and androgen-treated male chicks grow more quickly while females are either unaffected (Sockman et al., 2007) or experience reduced growth (Eising et al., 2003; Eising et al., 2003; Eising et al., 2003; Eising et al., 2003; Eising et al., 2003; Chen et al., 2010; Saino et al., 2006a).

In chickens, adult males are consistently larger than females; size differences are first apparent in embryos (Burke and Sharp, 1989), and reemerge by 3 weeks post hatch (Mignon-Grasteau et al., 1999). Experimental elevation of testosterone in chicken eggs had no effect on male embryonic growth or body mass of either sex at hatching, but suppressed female embryonic growth (Henry and Burke, 1999). Interestingly, blocking effects of endogenous androgens with flutamide (an androgen receptor antagonist) *in ovo* reduced growth in male chickens at 21 and 49 days post hatch, but did not alter growth of female chicks (Burke, 1996), despite enhancing muscle development in female embryos (Henry and Burke, 1999). For chickens, the consequences of exposure to elevated T described in these studies are not entirely consistent with the consequences of elevated yolk A4 described in the present study. While patterns of steroid receptor expression are likely important, the sex-dependent consequences of exposure to yolk A4 are likely to be driven by sex differences in steroid metabolism pathways, regulated by the expression and activity of steroidogenic enzymes.

3.3. Metabolism of A4 by enzymes

There are a number of steroidogenic enzymes that can act on A4 (Kanehisa et al., 2011), but there are two that convert it into highly bioactive molecules, both of which are present within the first week of avian embryonic development (Bruggeman et al., 2002; Shimada, 1998). The first is aromatase (p450arom), which converts A4 into estrone (and T into estradiol), and the second is 17β-HSD, which converts A4 into T (and estrone into estradiol) (Labrie et al., 1997). Thus, the same combination of enzymes can turn A4 into either T or (via estrone) estradiol, and turn T into either A4 or estradiol (Fig. 1) – this may lead to the assumption that A4 is functionally interchangeable with T. However, enzyme activity is highly dynamic, and can yield different products from these substrates. The proportions of yolk A4 that become T, estrone or estradiol depend not just on the relative quantities, isoforms and activities of the enzymes, but also on concentrations of the other steroids, which can compete for binding sites to inhibit or even reverse the direction of the reaction (Gibb and Lavoie, 1980; Labrie et al., 1997). Levels of activity differ between developmental stages, seasons, sexes and even different tissues within a sex (Forlano et al., 2006; Labrie et al., 1997; Simpson et al., 2002). In addition, activity of both enzymes can be rapidly modulated in response to local molecular cues (Cornil et al., 2012; Labrie et al., 1997). In fact, studies tracing the fates of labeled A4 and T consistently find different fates for these two steroids.

The only bird-specific studies to explore the metabolism of A4 quantified radiolabeled A4 derivatives from cultured embryonic chicken gonadal tissue. The fate of A4 varied between tissues and by age, and much of the A4 was 5-ß reduced; however, embryonic ovaries tended to produce both estradiol and estrone from A4 while testes did not (Imataka et al., 1988). While patterns of enzyme expression during development can be different from those during adulthood (Lauber and Lichtensteiger, 1994), studies in adult humans support a sex-dependent fate for A4. For example, administration of A4 can elevate T, A4 and DHT in young women (Bassindale, 2004) and in post-menopausal women, A4 also elevates estrone but does not change estradiol (Leder, 2002). In contrast, in men A4 increases estradiol but not T (Leder, 2002).

In addition to having the ability to convert A4 into different active steroids, embryos can metabolize steroids into bioactive metabolites like etiocholanonlone, which are poorly understood in birds (Paitz et al., 2010), or into less active forms (5-ß reduced androgens (Imataka, 1989), conjugated steroids (Engelhardt et al., 2009; Paitz et al., 2010)). Metabolism into water-soluble conjugates may reflect the ability of embryos to exert control over the developmental hormone milieu. For example, the sulfotransferase/sulfatase pathways may allow embryos to temporarily inactivate yolk steroids and then reactivate them as needed for development (Paitz and Bowden, 2008). Phase separation of yolk from day 7 embryos clearly indicates that even young embryos are metabolizing yolk A4 such that its derivatives are water soluble (Fig. 3). These aqueous derivatives, likely inactive conjugates, suggest that embryos are buffering levels of active yolk steroids. However the phenotypic consequences of elevated yolk A4 that we observed (Figs. 4–6) indicate that this buffering does not render variation in yolk steroid levels irrelevant.

The high levels of radioactivity in day 7 albumin and relatively low levels in yolk (compared to both albumin at day 7, and yolk + extraembryonic fluids at day 16; Fig. 2) is somewhat puzzling, and may either reflect rapid metabolism and diffusion of yolk steroids into water soluble conjugates, or incomplete injection of treatment into the yolk – we are not able to distinguish between these possibilities. Radioactivity was detected in all sampled tissue types at day 7 and day 16 of embryonic development (Fig. 2). The fate of A4 remains to be determined, but it will depend on enzyme expression and activity that is likely to differ by sex, tissues and developmental stage; regardless of the pathway there are clearly mechanisms by which it is being distributed throughout the body and metabolized (Figs. 2 and 3).

3.4. Potential role of estrogens

The fact that yolk A4 enhanced female growth but not male growth may be due to sex differences in rates of aromatization. Female chicken embryos and chicks produce estrogens at higher rates than males (Tanabe et al., 1986). In mammals, estrogens are involved in pre-pubertal growth and bone maturation (Grumbach, 2000), and estradiol promotes proliferation of chicken osteoblasts in vitro (Chen et al., 2010). Chicks from mothers with higher circulating levels of estradiol during egg laying grow more quickly, independently of egg size and sex (Engelhardt von et al., 2004). It is unclear if that difference in growth was a consequence of differences in levels of yolk estradiol, although maternal estrogens are deposited into eggs (Adkins-Regan et al., 2005). Studies experimentally altering exposure to yolk estradiol in birds are scarce, but in one study that assessed its effects in males, yolk estradiol altered digit ratio but not tarsus length in male pheasants (Saino et al., 2006b).

3.5. Comb

Chicken comb size is a sexually–selected, androgen dependent trait (Mukhutar and Kahn, 2012), and T manipulation can increase its growth in males even in the first week post-hatch (Astiningsih and Rogers, 2003), while experimentally elevated estrogen decreases comb size in male chickens (Stanton et al., 2001). In contrast, while T also promotes comb growth in females, comb size is positively correlated with circulating estrogen in developing females in some cases (Joseph et al., 2003), though this relationship

is not robust across lineages and experiments (Eitan et al., 1998). The most parsimonious explanation for the decreased comb size that we observed in A4 males (Fig. 5) is that A4 was aromatized to estrogen. Whether this is a tissue-specific or systemic fate for A4 is not clear. The lack of treatment effect in females does not provide much insight into the probable metabolic fate of A4 in females, other than that it was unlikely to substantially elevate T. If the difference between treatments persists into adulthood, it is in contrast with studies finding that sexually selected plumage traits were enhanced by exposure to elevated yolk T (Eising et al., 2006; Strasser and Schwabl, 2004), though this has not been demonstrated in all species tested (Müller and Eens, 2008).

3.6. Tonic immobility

Differences in behavioral phenotypes can generate differences in fitness (Groothuis and Carere, 2005; Smith and Blumstein, 2008). Tonic immobility – a state of remaining immobile after physical restraint ceases – is a stereotyped response to restraint that has been observed in many vertebrates, and is often interpreted as a measure of fearfulness (reviewed in (Heiblum et al., 1998)). Exposure to elevated yolk A4 appears to promote the development of a "less fearful" phenotype in chickens (Fig. 6). Several studies have investigated the effects of elevated yolk androgens on non-reproductive behavior. In passerines, elevated yolk T accelerated habituation to novel objects in canaries (Tobler and Sandell, 2007), and promoted activity and exploration in flycatchers (Ruuskanen and Laaksonen, 2010); both species showed stronger effects in males than in females. In Japanese quail, elevated yolk A4 enhanced a principle component of behavior referred as "activity" more strongly than T, while the principle component associated with fear was not affected by either treatment (Hegyi and Schwabl, 2010).

Effects of yolk T on tonic immobility behavior have been studied in quail at least twice, but results were inconsistent. One study showed that exposure to elevated yolk T increased tendency to become immobilized (Okuliarová et al., 2007), while another found that chicks from T-treated eggs were better at resisting immobility (Niall Daisley et al., 2005). Both age of chicks and quantity of T differed between the studies and it is not clear which is responsible for the disparate results. The behavioral effects of yolk A4 on day 3 chicks in this study were similar to those found by Daisley (2005) who found that yolk T reduced immobility and did not affect the sexes differently.

3.7. Persistence of early effects and exogenous hormone

The long-term consequences of A4 in this study are unknown; birds were months from adult body size or sexual maturation when the study was ended. There are examples where yolk steroids did not appear to affect chicks, but effects manifested in adults (Schwabl et al., 2011). However, alteration of the early endocrine environment often induces permanent changes in phenotype (Arnold and Breedlove, 1985; Bertram and Hanson, 2010; Fowden and Forhead, 2009), thus the effects observed here may either persist or reflect other underlying differences that will manifest later.

3.8. Conclusion

We established that while yolk A4 is not taken up from yolk differently by male and female embryos, it has different phenotypic consequences in the two sexes. Yolk A4 has been often (Gil et al., 2007; Groothuis and Schwabl, 2002; Hegyi et al., 2011; but not always; Hegyi and Schwabl, 2010) interpreted as being functionally equivalent to T and even experimentally elevated concurrently with T, potentially obscuring important biological variation. Similar lack of resolution may be generated by studies of yolk steroids that do not distinguish between male and female offspring (Hegyi and Schwabl, 2010; Pilz and Smith, 2004; Tschirren et al., 2005). The general increase in growth for females and the reduction of a sexually-selected trait in males suggests that, like many maternal effects, yolk A4 may impose trade-offs for mothers (J Marshall and Uller, 2007). These results also highlight the importance of investigating the sex-specific metabolic pathways of A4 during development (Groothuis and Schwabl, 2008). Efforts to distinguish the relative contributions of adaptation and constraint on both the deposition of steroids into egg yolk and offsprings' phenotypic responses to these hormones are ongoing, and are likely to require that consistent distinctions are made both among androgens and between sexes.

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