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Prenatal aromatase inhibition alters postnatal immunity in domestic chickens (*Gallus gallus*)

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ABSTRACT

In birds, exposure to exogenous testosterone during embryonic development can suppress measures of immune function; however, it is unclear whether these effects are due to direct or indirect action via aromatization. Estradiol (E_2) is synthesized from testosterone by the enzyme aromatase, and this conversion is a necessary step in many signaling pathways that are ostensibly testosterone-dependent. Many lines of evidence in mammals indicate that E_2 can affect immune function. We tested the hypothesis that some of the immunomodulatory effects observed in response to *in ovo* testosterone exposure in birds are mediated by conversion to E_2 by aromatase, by using fadrozole to inhibit aromatization of endogenous testosterone during a crucial period of embryonic immune system development in domestic chickens (*Gallus gallus*). We then measured total IgY antibody count, response to PHA challenge, mass of thymus and bursa of Fabricius, and plasma testosterone post-hatch on days 3 and 18. Because testosterone has a reputation for immunosuppression, we predicted that if modulation of an immune measure by testosterone is dependent on aromatization, then inhibition of estrogen production by fadrozole treatment would lead to elevated measures of that parameter. Conversely, if testosterone inhibits an immune measure directly, then fadrozole treatment would likely not alter that parameter. Fadrozole treatment reduced circulating E_2 in female embryos, but had no effect on males or on testosterone in either sex. Fadrozole-treated chicks had decreased day 3 plasma IgY antibody titers and a strong trend towards increased day 18 thymic mass. Furthermore, fadrozole treatment generated a positive relationship between testosterone and thymic mass in males, and tended to increase day 18 IgY levels for a given bursal mass in females. There was no effect on PHA response, bursal mass, or plasma testosterone at either age post-hatch. The alteration of several indicators of immune function in fadrozole-treated chicks implicates aromatization as a relevant pathway through which developmental exposure to testosterone can affect immunity in birds.

1. Introduction

The vertebrate immune system is responsible for defending the body from a variety of external threats and must be tightly regulated to optimize protection from disease. Investment in immune defenses begins as early as oocyte formation, when the mother deposits maternal antibodies, which will form the basis of embryonic immunity, into the egg (Ulmer-Franco, 2012). Of the multiple environmental and genetic factors that influence immune function, differential hormonal exposure is a prominent source of immunological variability (Olsen and Kovacs, 1996). While variation in hormone levels can transiently affect immune function in adults, hormone-mediated actions during development have the potential to permanently alter the trajectory of an individual's immune phenotype (Navara and Mendonca, 2008).

Of the hormones thought to influence immune development, testosterone has a reputation as a potent immunomodulator, and has long been described as a general inhibitor of immune activity (Roberts et al., 2004; Folstad and Karter, 1992). Testosterone exposure can suppress functionality of both B lymphocytes (antibody-producing cells) and T lymphocytes (regulators of cell-mediated immunity) *in vitro* (Cunningham and Gilkeson, 2011). In addition, a variety of vertebrate taxa exhibit decreased immune activity when treated with androgens. For instance, in adult mammals, androgen exposure causes apoptosis in cells of the thymus (the site of T cell maturation), as well as in circulating T cells and macrophages (Olsen and Kovacs, 1996). Studies of testosterone exposure during early development have reported similar effects. Experiments exploring potential trade-offs generated by variation in yolk testosterone levels have injected testosterone into eggs and

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found an array of effects on immune function, ranging from decreased post-hatch T cell concentrations and suppressed T cell-mediated responses to phytohemagglutinin (PHA), a general irritant and T cell mitogen (Andersson et al., 2004; Groothuis and Ros, 2005; Navara et al., 2005; Muller et al., 2005), to mixed effects where some immune measures were attenuated (pro-inflammatory cytokines) while others were increased (non-specific antibody response to LPS) (Kankova et al., 2018). In chickens, individuals from eggs treated with high doses of testosterone propionate show depressed antibody levels, as well as reduced weight of the bursa of Fabricius, an exclusively avian organ responsible for B-cell development and acquired immunity (Glick, 1961; Norton and Wira, 1977; Hirota et al., 1976). Thus, in birds, early administration of testosterone can inhibit several types of immune cells and immune cell generation, and these effects can persist after hatching.

1.1. Aromatase and Estradiol

Despite evidence for testosterone-induced immunosuppression, our mechanistic understanding of testosterone's complex role in immunity remains incomplete. Cytochrome P450 aromatase is an enzyme that converts androgens to estrogens, including converting androstenedione to estrone and testosterone to 17 β -estradiol (E_2) – all E_2 must be derived either from testosterone or from androstenedione via estrone as an intermediate, with both pathways requiring aromatization (Simpson et al., 2002). In addition, local conversion of testosterone to E_2 by aromatase is a key step in many signaling pathways purported to be testosterone-dependent. For instance, copulatory behavior in male quail requires aromatization of testosterone (Watson and Adkins-Regan, 1989) while in song sparrows, display of male territorial aggression during the non-breeding season are similarly dependent on the activity of aromatase (Soma et al., 2000). Experiments such as these suggest that many of the effects of testosterone are not regulated via direct action of androgen on androgen receptors but instead are mediated by aromatization to E_2 ; thus aromatization may also play a crucial role in aspects of putatively testosterone-dependent regulation of immunity.

The role of aromatization in immunomodulation has been studied extensively within the biomedical field (Subramanian et al., 2008; Niravath, 2013; Tanriverdi et al., 2003) and the prospect of immunosuppression by E_2 is frequently discussed in the context of xenoestrogens and other endocrine disruptors (Chalubinski and Kowalski, 2006; Razia et al., 2006; Markman et al., 2008). However, this pathway is often overlooked in other contexts, despite the long-standing knowledge that maternal testosterone can induce immunosuppression and acknowledgment of aromatization as a potential fate of testosterone (Owen-Ashley et al., 2004). Estrogens affect many components of the immune system, upregulating some and down-regulating others (Klein and Flanagan, 2016). Like testosterone, E_2 inhibits the T cell mediated response to PHA *in vitro* and causes dose-dependent thymic atrophy in mice (Ablyn et al., 1974; Wyle and Kent, 1977; Rijhsinghani et al., 1996; Okasha et al., 2001; Staples et al., 1999; Tai et al., 2008). Similarly, E_2 downregulates B cell lymphopoiesis in humans (Hill et al., 2011). The few existing studies in birds are largely restricted to adult or juveniles, and have found that E_2 tends to depress cell-mediated immunity, although reported effects on humoral immunity have been inconsistent (Owen-Ashley et al., 2004). Similarly, E_2 administration to chicken eggs on day 14 of incubation caused a suppressed antibody response to killed *Brucella abortus* 2–4 weeks after hatching, as well as a bursal weight reduction (Kondo et al., 2004). Thus, in birds there are similarities between the immunomodulatory effects of testosterone and E_2 that may implicate aromatization as a mechanism through which developmental exposure to testosterone acts on the immune system.

However, given the immune enhancing effects of E_2 on multiple immune traits in mammals, immunomodulatory effects of sex hormones in birds are likely to be trait-specific as well as variable across taxa and window of exposure (e.g. embryonic development vs adulthood) (Fish,

2008; Giefing-Kröll et al., 2015; Ahmed et al., 2010). Studies with non-aromatizable androgens such as DHT (dihydrotestosterone) or weakly-aromatizable androgens such as 19-nortestosterone (NorT) have provided support for the proposition that the role of aromatization in avian immunity is trait-specific. For instance, treatment of immature broiler chicks with E_2 or testosterone resulted in a decline in leukocytes, lymphocytes, and bursal weight, while administration of DHT did not (Al-Afaeq and Homeida, 1998). Similarly, while implantation of ca-ponized chickens with either testosterone or NorT implants both caused bursal atrophy, testosterone resulted in a reduction of spleen weight as well as PHA response, while NorT administration increased these immune metrics (Chen et al., 2010). While the importance of aromatization in immune regulation has been acknowledged in mammals (Martin, 2000) and in avian adults and juveniles, to our knowledge no prior studies have explored the role of aromatization during embryonic immune system development in birds, despite frequent study of the effects of exogenous testosterone on immune function in developing birds.

The purpose of our study was to determine whether conversion of endogenous androgens to estrogens is involved in regulating development of immune function in birds. We tested this by manipulating aromatase activity during development by exposing chicken embryos to an aromatase inhibitor, fadrozole, during a critical window for immune tissue development. For each immune trait measured, we consider three potential hypotheses. The first hypothesis asserts that the reduction in immune function documented in birds in response to embryonic testosterone administration is due (at least in part) to aromatization of androgens to estrogens. According to this hypothesis, aromatase inhibition *in ovo* would result in elevated immune activity relative to controls. The second hypothesis asserts that aromatization is not involved in regulating immune activity of developing birds. Therefore, according to this hypothesis exposure to an aromatase inhibitor a) would not change immune activity, or b) may actually decrease immune activity relative to controls if testosterone is immunosuppressive and inhibition of aromatase causes a buildup of testosterone due to lack of negative feedback in the hypothalamus. The third hypothesis states that estrogens enhance immunity rather than suppressing it, and thus aromatization induces a regulatory transition to greater immune activity. Under this hypothesis, aromatase inhibition would lead to reduced immunity (similar to the second hypothesis) but in this case due to the absence of estrogen receptor (ER) stimulation rather than an increase in androgen receptor (AR) signaling. Notably, the role of aromatization in immunity need not be unidirectional; the influence of AR and ER signaling may be trait-specific, and thus each metric of immune physiology or function should be considered separately.

2. Methods

2.1. Immune traits

We measured several traits representing different branches of the immune system. To measure acquired immunity, plasma IgY antibody levels were assayed at three points during development. IgY is the major immunoglobulin in birds, and is functionally similar to mammalian IgG antibody. Maternal IgY is deposited in egg yolk and is selectively transferred to the embryo via an IgY receptor (FcRY) embedded in the yolk cell membrane (Tesar et al., 2008). This maternally derived IgY acts as the basis of acquired immunity for the developing chick until it is able to produce its own at approximately day 6 post-hatch (Hamal et al., 2006). Because all IgY present before post-hatch day 6 is maternally derived, IgY titers before this point in development reflect uptake or retention of maternal antibodies. After day 6, IgY levels reflect a mixture of maternally derived and endogenous immunoglobulin. By day 14, maternal IgY levels are undetectable and all IgY is synthesized endogenously (Hamal et al., 2006). Previously, E_2 administration to hens has been shown to increase deposition of

maternal IgY into egg yolk (Barua et al., 2000). The mass of the bursa of Fabricius (hereafter “bursa”) was also recorded as a potential indicator of B lymphocyte generation, as bursal mass and antibody production are correlated (Glick, 1956; Sadler and Glick, 1962). Previous studies have produced mixed results with respect to hormonal influence on bursal mass: Al-Afaleq et al., documented a reduction in bursal weight due to testosterone and E₂ but not DHT (Al-Afaleq and Homeida, 1998), while Quinn et al observed an increase in the same metric due to developmental E₂ exposure (Quinn et al., 2009).

To assess T cell mediated immunity, swelling in response to PHA injection was measured (Hasselquist and Nilsson, 2012). A study by Chen et al. previously demonstrated that the PHA response was increased by non-aromatizable androgens but reduced by testosterone (Chen et al., 2010). Additionally, thymic mass was recorded as an immune metric as both testosterone and E₂ have been shown to induce thymic atrophy in mammals, with aromatase inhibition preventing this process (Staples et al., 1999; Greenstein et al., 1992).

All procedures were approved by Bucknell University's IACUC.

2.2. Eggs, fadrozole injection and validation

All eggs were fertilized, unincubated, White Leghorn domestic chicken eggs (*Gallus gallus*) from Moyer's Chicks in Quakertown, PA. They were incubated at 37.5 °C and 50% humidity in an OVA-Easy Advance incubator (Brinsea, Titusville, FL, USA) with a 90-minute turn cycle. We used two sets of eggs: One with which to validate the effectiveness of fadrozole in embryonic chickens (embryos were euthanized at day 15 of incubation for blood collection), and one in which chicks were reared to day 14 post-hatch. For both, eggs were sorted into control and fadrozole groups to balance the distribution of mass, and on day 13 of incubation 0.1 mg of fadrozole hydrochloride (Sigma-Aldrich, St. Louis, MO, USA; F3806) dissolved in 0.1 mL of 0.9% NaCl saline solution or saline only was injected in the air sac using a ½ inch, 27 g needle as previously described (Abinawanto and Saito, 1997; Yang et al., 2008).

Fadrozole is a non-steroidal aromatase inhibitor that reduces aromatase activity to near undetectable levels, and thus prevents any endogenous androgens from being converted into estrogens (Yue and Brodie, 1997). The dose of fadrozole was selected because it eliminates aromatase activity without reducing hatchability in chickens (Yang et al., 2008). To our knowledge, there are no studies that measure the elimination time of a single dose of fadrozole from an egg. However, administration of 0.1 mg of fadrozole (the same dose used in this study) to White Leghorn chicken eggs at day 5 of incubation induced full sex reversal in developing females, demonstrating that a single dose during development can have lasting effects on physiology (Abinawanto and Saito, 1997). The timing of fadrozole injection was selected based on studies showing that lymphoid stem cells have fully colonized the bursal primordium in chickens by day 14 of incubation, and that estrogen receptor expression in embryonic bursa and endogenously-derived plasma testosterone levels in both sexes are at a maximum at this age (Houssaint et al., 1976; Kondo et al., 2004; Woods et al., 1975). These studies suggest that this window of time is critical for estrogen signaling in developing immune tissues, and thus, if estrogens affect immune system development, fadrozole administration is likely to have pronounced effects during this period.

For the fadrozole validation, 97 eggs were distributed between treatments and injected on day 13 of incubation; egg mass did not differ between treatments (control: 56.96 ± 4.32 g, fadrozole: 56.71 ± 3.66 g). The 80 surviving embryos were bled and euthanized on day 15 of incubation. The egg was broken open exposing the embryo and its blood vessels, and a needle was used to puncture a major visible blood vessel on the yolk sac. Blood was collected with capillary tubes, separated by centrifugation, and placed in the -20 °C freezer. Genetic sex of each of the embryos was determined as described in section 2.6. Because plasma volumes were limited (20–200 µL), the samples were

allocated to either testosterone or E₂ groups, distributed evenly by sex and treatment.

2.3. Chicks

To determine the influence of transient *in ovo* fadrozole exposure on post-hatch immune measures, a separate batch of 80 fertilized, unincubated White Leghorn domestic chicken (*Gallus gallus*) eggs were sorted into control and fadrozole groups; egg mass did not differ between treatments (control: 44.9 ± 0.8 g; fadrozole: 45.3 ± 0.7 g). On day 13 of embryonic development, the eggs were injected with either control or fadrozole solutions in the same manner as described above.

On day 18 pre-hatch, eggs were transferred to a 1550 Hatcher (GQF, Savannah, GA, USA) at 36.7 °C and 60% humidity with no rotation. Chicks hatched on day 21 of incubation. Hatchlings were sexed based on feather structure (chickens of this strain are feather-sexable), banded in the wing web, weighed, and transferred to a brooder maintained at 35 °C. 71 (88.75%) chicks hatched over a period of 32 h. Chicks that had not hatched by 24 h following the first round of hatching were excluded from the experiment to minimize variability associated with hatching date, leaving a total sample of 58 chicks (16 control female, 16 control male, 12 fadrozole female, 14 fadrozole male). Once all experimental chicks had hatched, they were redistributed such that sex and treatment were balanced across three brooders. Chicks were given food and water ad libitum and kept on a 12:12 L:D photoperiod. Chicks were weighed every three days at 9 AM when the lights were turned on. The temperature was reduced by 3 °C each week and maintained constant once it reached 30 °C. All data were collected and samples analyzed by investigators blind to treatment.

2.4. PHA challenge

On day 3, day 13, and day 18 post-hatch, blood samples were drawn from the alar vein, using ½ inch, 26 g needles and capillary tubes. Samples were kept on ice and separated by centrifugation within an hour of collection; plasma was removed and stored at -20 °C until use in assays.

On day 14 post-hatch, a subset of chicks (36, distributed evenly across sex and treatment) were injected in the wing web of the unbanded wing with 0.1 mg of PHA (Sigma-Aldrich, St. Louis, MO, USA; L8754) dissolved in 0.06 mL of phosphate buffered saline (PBS), or with PBS alone, following web thickness measurement with a digital micrometer according to established protocols (Martin et al., 2006). Three measurements were taken and the average was used in calculations. Immediately following initial wing web thickness measurements, a ½ inch, 27 g needle tip on a 100 µL disposable syringe was used to slowly inject the PHA solution into the site of measurement. Nine chicks from each sex and treatment group were injected. 24 h later (± 1 h), the site of injection was measured again as described above. PHA injection did not influence any of the other metrics that we measured.

2.5. Immune tissue

On day 18 post-hatch, following a final blood sample collection, chicks were euthanized by decapitation. The bursa of Fabricius and thymus were extracted and weighed, and diagonal tarsus length was measured using a digital caliper (VWR, Radnor, PA, USA). Bursal mass and thymic mass were corrected for body size by dividing by overall body mass (as in Kondo et al., 2004).

2.6. Sex determination

Sex was confirmed both by visual inspection of gonads and by genetic sexing (Abinawanto and Saito, 1997). Genetic sex was determined with PCR and gel electrophoresis using AmplitaqGold PCR master mix (Applied Biosystems, Waltham, MA, USA; L00192), primers AvianSex

2550F (GTTACTGATTCGTCTACGAGA) and AvianSex2718R (ATTGAAATGATCCAGTGCTTG) (Fridolfsson and Ellegren, 1999), and 2.5% high-resolution agarose gels, which show one band for males and two for females. One fadrozole-treated chick demonstrated potential sex-reversal: it was initially identified as male based on feathers and gonads, but showed two bands in both of two rounds of genetic sexing. This chick was treated as female for subsequent analyses, though excluding this individual did not alter outcomes.

2.7. IgY content analysis

Plasma IgY content was determined using a Chicken IgG ELISA Quantification Set (Bethyl, Montgomery, TX, USA; E30-104-25). Dilutions of 1:40000 were consistently in the center of the standard curve and thus samples were analyzed in duplicate at this dilution ratio. Treatment groups were distributed equally across 5 plates with samples from the same chick kept on the same plate. Inter-assay variation based on an internal standard was 15.6%, while intra-assay variation for duplicate samples was typically in the 0–5% range ($\mu = 2.7 \pm 2.5\%$).

2.8. Plasma steroids

2.8.1. Embryos

Because of the high lipid content of embryonic plasma, steroids were extracted from embryonic plasma samples with dichloromethane following protocols described in (Moore et al., 2002), and recoveries were calculated from tritiated corticosterone (2000 cpm/sample). Each sample value was corrected for its individual recovery. Testosterone and E₂ were quantified using Expanded Range Salivary Testosterone Enzyme Immunoassay kit (Salimetrics, State College, PA, USA 1-2312) and High Sensitivity Salivary 17 β E₂ Enzyme Immunoassay kit (Salimetrics, State College, PA, USA 1-4702), both with detection limits of 1 pg/mL. Serial dilutions were used to confirm parallelism with the standard curve for the extracted steroids. There was a negative relationship between plasma volume and final concentration, which suggests that samples from low plasma volumes had inflated final concentrations. Therefore, samples with < 100 μ L were excluded from all further analyses, eliminating this relationship and resulting in final sample sizes of 36 for both E₂ and testosterone. Testosterone was measured in duplicate but there was not sufficient sample to assay E₂ in duplicate. The average % CV for the testosterone assay was 6.05%.

2.8.2. Chicks

The same Salimetrics kit was used to quantify testosterone in chick plasma on day 3 and day 18 post-hatch. Because this kit has been used successfully to quantify plasma testosterone in several avian species, and serial dilutions of unextracted chick plasma were parallel with the standard curve (Washburn et al., 2007; Marteinson et al., 2011), we did not run an extraction. Samples were run across 3 plates, and samples from the same individual at both ages were run on the same plate to reduce inter-assay variation. Inter-assay variation based on high and low controls were 6.7% and 3.4% respectively, and intra-assay variation for duplicate samples was $5.2 \pm 5.1\%$. Testosterone was not detectable in some plasma samples; for these, we set values to 1 pg/mL, the lower detection limit for the assay, but also ran the statistical analyses without the undetectable samples included. We were unable to detect plasma E₂ with the small remaining quantities of plasma.

2.9. Statistical analyses

We validated the effect of fadrozole on circulating E₂ and testosterone in embryos that were sampled at day 15 of incubation (details above). We analyzed the E₂ and testosterone data using generalized linear models. All models included sex, treatment (fadrozole or control), and their interaction, to determine if the effect of treatment on circulating hormone concentrations varied depending on the sex of the

embryo. We used post-hoc Tukey tests of least squares means to contrast hormone concentrations among groups.

We conducted all of our main analyses in R (version 3.0.2). For all of our analyses, we selected the models that best fit the data using model comparison with Akaike's Information Criterion corrected for small sample size (AICc) using the *dredge* command in the R package *MuMIn* (Symonds and Moussalli, 2011). When more than one model was within 2 AICc of the top model, we report model-averaged effects across the top models using the *model.avg* command, also in *MuMIn* (Posada and Buckley, 2004; Burnham and Anderson, 2004). However, if only one model was best fit (i.e., no other models within 2 AICc), we only report results from that model. For all analyses, we initially ran linear mixed-effects models (LMEs, using the command *lmer* in the package *lme4*) and assessed the effect of inclusion of a random grouping effect of brooder (i.e., the compartment in which hatchlings were housed – hatchlings were housed in 3 different brooders) by comparing the AICc of the full model containing or lacking this random effect. If the AICc was > 2 lower when this random effect was included, we retained it in the full model that was then used in model selection for fixed effects. For all models analyzing response metrics that were estimated more than once within individuals, we included individual identity as a random effect. For models where a random effect was not retained (because individuals were only sampled once, and brooder did not improve model fit), we used generalized linear models (GLMs) instead of LMEs. We checked the fit of all top models by visually examining plotted relationships between predicted values and model residuals, and fixed effects and residuals, as well as by testing for normal distribution of residuals.

To determine if the treatment influenced any of the response metrics (production or uptake of IgY, bursal or thymic mass, post-hatch testosterone, or the PHA response) we conducted separate LMEs with the full model containing treatment (control or fadrozole-treated), sex, body mass, and age (3, 13, or 18 days old) as fixed effects where appropriate. We log-transformed IgY and testosterone concentrations to fit a normal distribution. We corrected bursal mass and thymic mass by dividing by body mass, so we did not include body mass in these models. We also included the interactions of treatment by age and treatment by sex. If fadrozole treatment influenced any of the response variables, the treatment main effect or treatment by age interaction effect would be significant.

We also conducted an additional analysis to determine if treatment with fadrozole influenced the efficiency of IgY production by the bursa. We used an LME with log-transformed IgY measured at 18 days old as the dependent variable. The full model included treatment, size-corrected bursa (as described above), sex, and body mass, as well as the interactions of bursa by treatment, bursa by sex, treatment by sex, and treatment by sex by bursa as fixed effects. If treatment influenced the production of IgY by the bursa, we would predict that the relationship between bursa mass and IgY would differ for fadrozole-treated birds (i.e., a significant bursa by treatment interaction effect).

Finally, to determine if treatment with fadrozole influenced the relationship between testosterone and thymic mass, we conducted an LME with size-corrected thymic mass as the dependent variable. The full model included treatment, log-transformed testosterone (measured at 18 days old), sex, and all possible 2-way interactions and the 3-way interaction as fixed effects. If fadrozole treatment influences the effect of testosterone on thymic mass, we would predict that the relationship between testosterone and thymic mass would differ for fadrozole-treated birds (i.e., a significant treatment by testosterone effect).

3. Results

3.1. Hatching & growth

All chicks included in the experiment hatched within 24 h of each other. Hatching success did not differ between treatments (90% (36 out

Fig. 1. Plasma (A) testosterone and (B) E_2 concentrations at day 15 of incubation (2 days post-injection) for control and fadrozole-treated birds. For each boxplot, the middle line represents the median, the bottom and top hinges represent the 25th and 75th percentiles, respectively, and the whiskers extend to the furthest outliers still within 1.5x the interquartile range from the nearest hinge (25th or 75th percentile).

of 40 eggs) for control and 85% (34 out of 40 eggs) for fadrozole-treated chicks; ($\chi^2(1, N = 80) = 0.125, p = 0.723$). Fadrozole treatment did not affect hatch mass ($F_{1,56} = 0.363, p = 0.549$), body mass gain ($F_{1,56} = 0.209, p = 0.65$), or day 18 tarsus length ($F_{1,55} = 0.63, p = 0.431$).

3.2. Fadrozole activity validation

Testosterone data were normally distributed, so raw data were analyzed. Fadrozole treatment did not affect circulating testosterone concentrations, regardless of embryo sex (sex by treatment interaction: $F_{1,34} = 0.08, p = 0.78$, main treatment effect: $F_{1,34} < 0.001, p = 0.99$; Fig. 1A). Testosterone tended to be higher in female embryos relative to males, regardless of treatment group (Tukey test, $z = 1.84, p = 0.067$). E_2 data were log-transformed prior to analysis to meet assumptions of normality. On day 15 of incubation (2 days post-injection), the effect of treatment with fadrozole on E_2 concentrations depended on sex of the embryo (sex by treatment interaction: $F_{1,32} = 6.63, p = 0.015$; Fig. 1B). Fadrozole-treated females had lower circulating E_2 than control females (Tukey test, $z = 3.69, p = 0.0002$), but E_2 did not differ among control and fadrozole-treated males (Tukey test, $z = -0.06, p = 0.95$).

3.3. Plasma IgY concentrations

Inclusion of brooder as a random effect improved model fit ($\sigma^2 = 0.002 \pm 0.04$; all random effects reported as estimated variance $\pm$ standard deviation), so model comparison was conducted with an LME including both brooder and individual identity ($\sigma^2 = 0.004 \pm 0.06$) as random effects (because IgY was estimated in the same individuals three times). The 6 best-fit models for explaining variation in IgY concentrations retained age of the individual, treatment, body mass, sex, and an interaction of treatment by age in various combinations (Table 1). In model-averaged estimates, only age, treatment, and the treatment by age interaction were related to IgY concentrations. Overall, IgY declined with age ($\beta = -0.054, p < 0.001$) and was lower in fadrozole-treated birds versus controls ($\beta = -0.071, p = 0.018$), but this difference diminished with age ($\beta = 0.004, p = 0.037$; Fig. 2).

Table 1

Predictors of IgY concentrations estimated using model averaging for the 6 best-fit models (all within 2 AICc of the top model).

Fixed effect	Estimate	SE	z	P
Age	-0.054	0.004	12.44	< 0.001
Treatment	-0.071	0.030	2.37	0.018
Body mass	0.001	0.001	1.42	0.156
Sex	-0.018	0.022	0.81	0.417
Treatment by age	0.004	0.002	2.09	0.037

3.4. Bursal mass

Inclusion of brooder as a random effect did not improve model fit ($\sigma^2 < 0.0001 \pm 0.00$), so a GLM without any random effect was used for model selection. The best-fit model was the null model, but 3 other models were similarly well fit to the data as the null (i.e., within 2 AICc). These models individually included fixed effects of phytohemagglutinin (PHA) status (whether or not the bird was injected with PHA), treatment, or sex. However, in model averaging, none of the effects of these factors were significant predictors of bursa mass (all $p > 0.3$). Thus, treatment with fadrozole did not affect size-corrected bursal mass.

3.5. Thymic mass

Inclusion of brooder as a random effect did improve model fit ($\sigma^2 < 0.0001 \pm 0.00$), so model selection was conducted using an LME with this random effect retained. The 3 best-fit models contained sex, treatment, and their interaction (Table 2). Model averaged effects reveal that, overall, males tended to have lower size-corrected thymic mass ($\beta = -0.001, p = 0.083$), and that fadrozole-treated birds tended to have higher size-corrected thymic mass ($\beta = 0.001, p = 0.056$; Fig. 3). The interaction term was not significant.

3.6. Post-Hatch plasma testosterone concentration

Inclusion of brooder as a random effect did not improve model fit ($\sigma^2 = 0.00 \pm 0.00$), so model selection was conducted using an LME with only individual identity as a random effect ($\sigma^2 = 0.00 \pm 0.00$; retained because testosterone was measured twice in each individual). The 3 best fit models retained age, body mass, and sex; treatment was

Fig. 2. Plasma IgY antibody concentrations for control and fadrozole-treated chicks. For each boxplot, the middle line represents the median, the bottom and top hinges represent the 25th and 75th percentiles, respectively, and the whiskers extend to the furthest outliers still within 1.5x the interquartile range from the nearest hinge (25th or 75th percentile).

Table 2
Predictors of size-corrected thymic mass estimated using model averaging for the 3 best-fit models (all within 2 AICc of the top model).

Fixed effect	Estimate	SE	z	p
Treatment	0.001	0.0003	1.91	0.056
Sex	-0.001	0.0003	1.73	0.083
Sex by treatment	-0.001	0.0006	0.90	0.366

not retained in any of the top models. Model-averaged estimates from the best-fit models indicate that testosterone was higher in males than females ($\beta = 0.856, p < 0.001$) and declined with age ($\beta = -0.151, p = 0.045$). The effect of body mass was not significant ($\beta = 0.007, p = 0.202$). Results are similar if birds without detectable testosterone concentrations are excluded from the analyses; however, the decline with age is dampened and no longer significant ($\beta = -0.026, p = 0.186$). Thus, treatment with fadrozole did not affect circulating testosterone concentrations in 3- or 18-day old birds (Fig. 4).

Fig. 3. Thymic mass (corrected for body mass) of control and fadrozole-treated chicks. For each boxplot, the middle line represents the median, the bottom and top hinges represent the 25th and 75th percentiles, respectively, and the whiskers extend to the furthest outliers still within 1.5x the interquartile range from the nearest hinge (25th or 75th percentile).

3.7. IgY-Bursa relationship

The inclusion of brooder as a random effect ($\sigma^2 = 0.002 \pm 0.04$) did not improve model fit, so model selection was conducted with a GLM. The 3 best-fit models retained treatment, size-corrected bursal mass, sex, a sex by bursal mass interaction, a treatment by bursa interaction, and a sex by treatment interaction effect (Table 3). Model averaging revealed both a sex-dependent ($\beta = -104.5, p < 0.001$) and a treatment-dependent relationship between bursal mass and IgY ($\beta = 67.14, p = 0.027$). Within females, fadrozole exposure caused the positive relationship between bursal mass and day 18 IgY to increase in slope, which led to elevated IgY levels for a given bursal mass relative to controls ($R^2 = 0.40, p = 0.026$). Conversely, control males exhibited a negative correlation between bursal mass and day 18 IgY, with fadrozole treatment tending to dampen the relationship (Fig. 5).

3.8. Testosterone-thymus relationship

Inclusion of brooder as a random effect did improve model fit ($\sigma^2 < 0.0001 \pm 0.00$), so model selection was conducted using a LME with this random effect. The 5 best-fit models retained log-testosterone,

Fig. 4. Plasma testosterone levels of control and fadrozole-treated chicks at day 3 and day 18 post-hatch. Only individuals with detectable testosterone levels are shown, though relative levels by sex and treatment are unchanged if undetectable samples are included. For each boxplot, the middle line represents the median, the bottom and top hinges represent the 25th and 75th percentiles, respectively, and the whiskers extend to the furthest outliers still within 1.5x the interquartile range from the nearest hinge (25th or 75th percentile).

Table 3
Predictors of day 18 IgY concentrations measured estimated using model averaging for the 3 best-fit models (all within 2 AICc of the top model).

Fixed effect	Estimate	SE	z	P
Treatment	−0.355	0.178	1.95	0.051
Sex	0.614	0.182	3.30	< 0.001
Bursa mass	36.17	25.66	1.38	0.168
Bursa by sex	−104.5	30.83	3.32	< 0.001
Bursa by treatment	67.14	29.58	2.22	0.027
Sex by treatment	−0.081	0.050	1.59	0.112

sex, treatment, a sex by treatment interaction effect, and a testosterone by treatment interaction effect (Table 4). Model averaging indicated that the interaction of log-testosterone by treatment and sex by treatment were both marginally significant. The majority of females had undetectable testosterone levels at day 18, precluding analysis of the relationship between testosterone and thymic mass. In control males, there was a negative relationship between plasma testosterone and size-corrected thymic mass. In contrast, fadrozole-treated males exhibited a positive relationship between plasma testosterone and size-corrected thymic mass (Fig. 6).

Table 4
Predictors of size-corrected thymic mass estimated using model averaging for the 5 best-fit models (all within 2 AICc of the top model).

Fixed effect	Estimate	SE	z	P
Treatment	0.001	0.0004	1.40	0.161
Sex	−0.001	0.0006	1.62	0.105
Testosterone	0.0003	0.0004	0.79	0.428
Testosterone by treatment	0.001	0.0006	2.03	0.043
Sex by treatment	−0.002	0.0009	1.99	0.047

3.9. Response to PHA

The inclusion of brooder as a random effect ($\sigma^2 = 0.00 \pm 0.00$) did not improve model fit, so model selection was conducted with a GLM. The null model was the best-fit model, but 3 other models were similarly well-fit to the data. These 3 models retained sex and body mass in various combinations; treatment was not included in any of the best-fit models. Model averaging across the 4 top models did not reveal any significant effects (all $p > 0.14$). Thus, treatment with fadrozole did not influence the wing-web swelling response to injection with PHA.

Fig. 5. Relationship between bursal mass and day 18 IgY levels by treatment for females (left) and males (right).

Fig. 6. Relationship between day 18 plasma testosterone levels and day 18 thymic mass for males by treatment.

4. Discussion

4.1. Summary

We show for the first time that prenatal inhibition of estrogen production alters aspects of post-natal immunity in birds. Furthermore, because estrogen synthesis was inhibited transiently during embryonic development, the differences observed in chicks suggest that even temporary changes in embryonic estrogen production can cause persistent changes in aspects of immune physiology, indicating an organizational role for estrogens on the avian immune system. Aromatase inhibition resulted in long-term changes to several immune measures but did not alter plasma testosterone levels in postnatal animals. Aromatase inhibition reduced Day 3 circulating IgY levels, increased day 18 thymic mass, and increased day 18 IgY levels for a given bursal mass in females. While thymic mass in control males was negatively correlated with plasma testosterone titers, as expected (Mase and Oishi, 1991), the opposite was true of fadrozole-treated birds, with higher plasma testosterone titers being associated with larger thymuses. Because E_2 , but not testosterone, levels were significantly altered during the window of fadrozole activity, our results implicate E_2 deprivation as the likely agent of observed immune changes, and contribute to a growing body of literature suggesting that developmental estrogens may be as important as androgens in shaping the avian immune system (Ahmed, 2000; Roberts et al., 2004; Owen-Ashley et al., 2004; Al-Afalek and Homeida, 1998).

4.2. Fadrozole activity validation

Fadrozole treatment on day 13 pre-hatch significantly decreased E_2 in females on day 15 of incubation, with treated chicks exhibiting mean plasma levels of about half their control counterparts (Fig. 1B). Furthermore, treatment did not influence plasma testosterone levels at the same time point, likely due to the overall higher levels of endogenous testosterone relative to E_2 (Fig. 1A). As this developmental window represents a critical period for steroid hormone-mediated immune development, and because testosterone levels were unaffected by treatment, transient *in ovo* E_2 deprivation is implicated as the likely causal factor for immune effects observed at later time points in females.

For males, embryonic day 15 testosterone and E_2 levels were unaffected by fadrozole exposure (Figs. 2 and 3). It is unclear what sex-specific factors account for the markedly different response to aromatase inhibition at the time of measurement, with disparate fadrozole clearance profiles or differences in endogenous aromatase expression among the potential causes. Furthermore, because much of endogenous aromatase activity is highly localized within target tissues, circulating E_2 concentrations may not always reflect site-specific E_2 activity levels. There is some evidence to support this: in a 2013 study

of song acquisition in male songbirds in response to continuous exposure to the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) via cranial implant, Bailey et al. documented a significant decrease in E_2 within the hippocampus, but not in plasma levels (Bailey et al., 2013). Thus, while a change in circulating E_2 levels provides strong evidence for efficacious treatment, the absence of such change does not imply a lack of treatment effect, as the ATD-treated birds in Bailey's study exhibited striking changes to behavior and physiology, despite plasma E_2 levels remaining unchanged. However, as we are unable to conclusively demonstrate a reduction in E_2 exposure for males in this cohort, more caution must be applied in the interpretation of post-hatch results in male birds.

4.3. Endogenous IgY

Fadrozole exposure tended to increase day 18 IgY levels for a given bursal mass in females (Fig. 5). This relationship suggests that estrogens inhibit bursal IgY production and supports hypothesis one which states that downregulation of certain immune traits by testosterone is mediated by aromatization of androgens. Because maternal IgY levels become undetectable by day 14 post-hatch, day 18 IgY is entirely endogenous (Hamal et al., 2006). The fact that the effect of fadrozole on IgY levels varied from day 3 to day 18 also provides evidence that estrogens have different effects on different mechanisms within the immune system. In this case, estrogens may promote uptake of maternal IgY, perhaps via the FcRY receptor expression (see section 4.5) but inhibit endogenous IgY production after hatching (Hamal et al., 2006; Kondo et al., 2004).

4.4. Maternal IgY: Day 3 Post-Hatch

In ovo fadrozole exposure caused a reduction in Day 3 post-hatch plasma IgY concentration, supporting hypothesis three which asserts that aromatization induces a transition from downregulation to upregulation of certain immune measures (Fig. 2). As IgY is not synthesized endogenously until post-hatch day 6 in chickens, the IgY titers observed at day 3 represent maternal antibodies that have been retained in the developing chick (Hamal et al., 2006); thus these results are consistent with the finding of Barua et al. that administration of E_2 to hens results in an increase in IgY deposition into their eggs (Barua et al., 2000). Maternal IgY is transferred from the yolk to the embryo via a selective receptor (FcRY) embedded within the yolk membrane (Tesar et al., 2008). The observed decrease in IgY levels in response to developmental aromatase inhibition can therefore be explained by either a change in uptake (mediated either directly via alteration of FcRY expression or activity, or indirectly through some other means) or by upregulation of IgY catabolism or excretion. We are not able to distinguish between these possibilities in this study, but to our knowledge, this is the first study to demonstrate that inhibition of aromatase alters uptake or retention of maternal IgY antibody. If expression or activity of receptors for maternal antibodies can be directly influenced by hormone exposure during prenatal development, it suggests a potential interaction among different maternal effects (endocrine versus immune). Variation in IgY uptake could have far-reaching effects on the immune system because maternal IgY provides the basis of acquired immunity until well after hatching (Hamal et al., 2006). Thus variation in IgY uptake could introduce variation in the maturing immune system and in susceptibility to early infection.

4.5. Thymic mass

Fadrozole exposure tended to increase body mass-corrected thymic mass of 18 day old chicks in females (by about 15%; Fig. 3), suggesting that endogenous estrogens inhibit thymic growth for a given body size and therefore supporting hypothesis one which states that downregulation of certain immune traits by testosterone is mediated by

aromatization to E₂. Our results corroborate previous studies in mammals showing that the inhibitory actions of testosterone on thymic growth are mediated in part by conversion to E₂ (Greenstein et al., 1988), and studies in amphibians showing that E₂ treatment induces apoptosis of thymocytes (Quinde et al., 2014). It is difficult to decipher the fitness implications of a difference in thymic mass, because the size of the thymus does not always correspond with T cell populations (Hasselbalch et al., 1997). However, a change in thymic mass in response to inhibition of pre-natal estrogen production represents a potentially important alteration in immunological state.

4.6. PHA challenge

Fadrozole exposure had no effect on response to PHA. The PHA response challenge has long been used as proxy for T cell mediated immunity, and has become one of the most widely used ecoimmunological assays due to its simplicity and cost efficiency (Martin et al., 2006). However, the mechanics of PHA-induced inflammation generate a complex relationship between T cell activity and the PHA response. While PHA does indeed induce T lymphocyte proliferation, the downstream inflammatory response involves a host of other cells as well as regulatory factors, and it is ultimately macrophages, heterophils, and basophils that are the direct agents of swelling (Stadecker et al., 1977; McCorkle et al., 1980; Tella et al., 2008). For this reason, it has been suggested that the PHA challenge assay be considered a “multifaceted index of cutaneous immune activity” more so than a direct measure of T cell mediated immunocompetence (Martin et al., 2006).

4.7. Plasma testosterone

The lack of differences among treatments in day 3 and day 18 chick plasma testosterone levels (Fig. 4) suggests that the changes observed were mediated by organizational effects allowing for long-term alteration of physiology despite transient exposure to fadrozole (testosterone should be elevated in fadrozole-treated chicks if it were still inhibiting aromatase activity at those ages) (Sechman et al., 2003). Furthermore, the lack of change in testosterone observed *in ovo* in response to treatment, in contrast to the concomitant decrease in E₂ in female embryos, implicate E₂ as the probable agent of immunomodulation documented here. This assertion is bolstered by the literature, with a wealth of studies showing that manipulation of *in ovo* testosterone exposure in chickens produces inhibition of immune function (Glick, 1961, 1956; Hirota et al., 1976; Hillgarth and Wingfield, 1997; Navara and Mendonca, 2008; Norton and Wira, 1977). Thus even if fadrozole treatment had elevated testosterone in developing chicks, it would be unlikely that the measures of immune function that were elevated in the present study were driven directly by testosterone changes. In addition, studies in rats provide precedent for some of our results.

In one experiment, aging and young adult rats were orchidectomized and surgically implanted with testosterone or testosterone and the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD). The thymus naturally degenerates with age in rats, and the effect was accelerated in the testosterone only treatment. However, the testosterone + aromatase inhibitor treatment caused a restoration of the thymus, and even enlarged the gland in young intact rats, indicating that testosterone-dependent thymic degeneration is contingent on aromatization (Greenstein et al., 1992). Thus, our results, in conjunction with previous studies, strongly implicate aromatization as a pathway through which prenatal exposure to testosterone modulates immunity. If this is the case, then our current understanding of testosterone's role in the development of avian immunity is incomplete. Manipulation of testosterone either during development or in adulthood is a standard method for studying the role of androgens, yet without accounting for aromatization, it is impossible to distinguish between the effects of testosterone and those of one of its active metabolites, E₂.

4.8. Conclusion

We demonstrate that developmental aromatase inhibition alters measures of immune activity in chickens. Combined with the long-standing observations that exposure to testosterone generally results in suppression of immune metrics, and that estrogens are strongly immunomodulatory, our results suggest that aromatization plays a crucial role in the testosterone-mediated immunomodulation observed in developing birds, and that its role is immune metric-specific.

Experimental ablation alone is not sufficient to fully elucidate a hormone's role in a physiological process (Zera et al., 2007), and a more integrative investigation, including ablation and replacement experiments, manipulation with non-aromatizable androgens like dihydrotestosterone (DHT), molecular studies of estrogen receptors, and fitness studies, will be needed to disentangle the relative contribution of estrogens to the development of immune function in birds, and to understand the mechanisms by which variation in yolk testosterone (whether endogenous or experimentally generated) may induce trade-offs between the immune system and other aspects of phenotype. However, the present study provides valuable groundwork towards this end. Considering the paucity of data regarding estrogen-mediated immunomodulation in the context of physiological and behavioral ecology relative to its precursor, testosterone, future investigations into endocrine-immune interactions may benefit from increased investigation into the potential role of aromatization.

CRedit authorship contribution statement

J.W. Simkins: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **A.E. Joseph:** Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization. **F. Bonier:** Software, Formal analysis, Writing - review & editing. **Z.M. Benowitz-Fredericks:** Methodology, Software, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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