# Developmental programming of the adrenocortical stress response by yolk testosterone depends on sex and life history stage

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**ABSTRACT:**

Developmental exposure of embryos to maternal hormones such as testosterone (T) in the avian egg influences the expression of multiple traits, with certain effects being sex-specific and lasting into adulthood. This pleiotropy, sex dependency, and persistency may be the consequence of developmental programming of basic systemic processes like adrenocortical activity or metabolic rate. We investigated if experimentally increased *in ovo* exposure to T influences hypothalamus-pituitary-adrenal function, i.e. baseline and stress-induced corticosterone (Cort) secretion, and resting metabolic rate (RMR) of adult male and female House sparrows (*Passer domesticus*). In previous experiments with this passerine bird we demonstrated effects of embryonic T exposure on adult agonistic and sexual behavior and survival. Here we report that baseline Cort levels and the stress secretion profile of Cort are modified by *in ovo* T in a sex-specific and life-history stage dependent manner. Compared to controls, males from T-treated eggs had higher baseline Cort levels whereas females from T-treated eggs showed prolonged stress-induced Cort secretion during the reproductive but not the non-reproductive phase. Adult RMR was unaffected by *in ovo* T-treatment but correlated with integrated Cort stress secretion levels. We conclude that exposure of the embryo to T programs the hypothalamus-pituitary-adrenal axis in a sex-specific manner that in females depends, in expression, on reproductive state. The modified baseline Cort levels in males respectively stress-induced Cort levels in females may explain some of the long-lasting effects of maternal T in the egg on behavior and could be linked to previously observed reduced mortality of T-treated females.



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## INTRODUCTION

The developmental pathways from genes to phenotype include environmental and maternal non-genomic input which leads to variation in phenotypes (Gilbert and Epel, 2009; Monaghan, 2008). Maternally provisioned hormones represent such non-genomic input, contributing to the developmental hormonal milieu and modifying phenotype development in vertebrates (mammals: Dantzer et al., 2013; Dloniak et al., 2006; Meise et al., 2016; birds: Schwabl, 1993; Schwabl and Groothuis, 2010; Groothuis et al. 2019; Podmokla et al. 2018; fishes: Feist et al., 1990; Giesing et al., 2011; reptiles: Lovern and Wade, 2003; Paitz and Bowden, 2009; amphibians: Meylan et al., 2012). The effects of maternal hormones, such as androgens in the avian egg, on offspring traits can be expressed during early development (e.g. Schwabl, 1996; Schwabl et al., 2007), in juveniles (e.g. Schwabl 1993), and in adults (e.g. Eising et al., 2006; Hsu et al., 2016; Partecke and Schwabl, 2008; Rubolini et al. 2006; Ruskaanen et al., 2013; Schweitzer et al., 2013; Strasser and Schwabl, 2004). Multiple traits, ranging from growth to morphology, to physiology, to behavior can be influenced by a single hormone such as testosterone (Groothuis and Schwabl, 2008**;** Hsu et al., 2016; Parolini et al., 2017; Schwabl & Groothuis, 2010; Schweitzer et al.; 2013; Tobler et al., 2007; Treidel et al., 2013; Tschirren et al., 2007) and, on top of this pleiotropy (Dantzer and Swanson, 2017; Navara and Mendonça, 2008), effects can be sex-specific (e.g. Sockman et al., 2007; Tschirren, 2015).

The mechanisms underlying pleiotropy, sex-specificity, and persistency of hormonally mediated maternal effects remain unclear. Although maternal steroids may interact with the hormonal signaling processes associated with developmental organization of sex differences (Adkins-Regan, 2012; Carere and Balthazart, 2007) they likely also operate through mechanisms independent of and possibly well before sexual differentiation (Kumar et al., 2018; Schwabl and Groothuis, 2010). Pleiotropy may result from evolved hormonal integration of suites of traits and physiological tradeoff, or it may be the consequence of modification of fundamental systemic processes that impact the function of other functions (Groothuis and Schwabl, 2008). Here we investigated the effects of developmental exposure to T on two fundamental

organismal processes that are known to impact and modify many other systems, functions, and traits - the hypothalamus-pituitary-adrenal (HPA) axis (Crino et al., 2017; Hau and Goymann, 2015; Hau et al., 2016; Sapolsky et al., 2000) and metabolic rate (Biro and Stamps, 2010; Careau and Garland, 2012; Glazier, 2015; Mathot et al., 2015; Mathot and Dingemanse, 2015; Holtmann et al., 2017).

Vertebrate HPA activity is characterized by dynamic functional states. Baseline "tone" of HPA-regulated glucocorticoid secretion varies with changing energy requirements associated with the light/dark cycle, activity levels, and life history stage to maintain energy homeostasis (Landys et al., 2006; Sapolsky et al., 2000); rapid stimulation of the HPA axis in response to stressors leads to a fast, episodic increase of circulating glucocorticoid titers, redirecting physiology and behavior into an emergency life history state (Wingfield et al., 1998). Termination of the stress response and recovery from stress activation is mediated by negative feedback to down-regulate glucocorticoid secretion to baseline levels which can vary with sex, age, past stress experience, and genotype (e.g. Baugh et al., 2012; Gomez et al., 1998; Gormally and Romero, 2018; Novais et al., 2017; Sapolsky et al., 1986; Schmidt et al., 2012; Schwabl, 1995). Because baseline and stress-induced glucocorticoid levels influence many functions, from metabolism, immune defense, reproduction, and behavior, to risk of contracting disease (Hau and Goymann, 2015; Hau et al., 2016; Sapolsky et al., 1986; Vágási et al. 2018), the developmental programming of HPA activity could be a common denominator underlying the diverse long-lasting effects of maternal androgens. Previous research showed modification of plasma levels of corticosterone (Cort), the major avian glucocorticoid, by embryonic exposure to androgens in the American kestrel (*Falco sparverius*) where nestlings from androgen-treated eggs (testosterone plus androstenedione) showed higher Cort levels than those from control eggs (Sockman & Schwabl, 2001). Long-lasting effects of exposure to maternal androgens in the eggs on baseline Cort levels and the stress response of adults have, however, to the best of our knowledge, not been reported before.

Resting metabolic rate (RMR) reflects the energy requirements of basic cellular and organismal function (Hulbert and Else, 2000) and it varies, across species for example with pace of life (e.g. Wiersma et al., 2007) and, within species with sex, life history stage, and season (Aschoff and Pohl, 1970; McNab, 2012; Wikelski et al., 1999). Maternal hormonal modification of offspring metabolic rate could impact an offspring's phenotype by effects on development rate (Schwabl et al., 2007; Martin and Schwabl, 2008), scope of performance (Wiersma et al., 2007), and overall energy costs of living (Hulbert and Else, 2000; Speakman, 2000). Theoretical considerations and empirical studies suggest that individual differences in metabolic rate may lead to individual variation in behavior (e.g. Biro and Stamps, 2010; Careau et al., 2012; Holtmann et al., 2017; Killenn et al., 2013; Mathot et al., 2015) and impact aging, mortality, and fitness (Burton et al., 2011; Harmann, 1956; Perez-Campo *et al*.,1998). Experimentally elevated *in ovo* androgen exposure increased RMR in nestling (T treatment, Tobler et al., 2007) and adult zebra finches *Taeniopygia guttata* (Nilsson et al., 2011; same individuals as in Tobler et al., 2007, sexes not identified) and in both sexes of adult Pied flycatchers *Ficedula hypoleuca* (combined T plus androstenedione treatment, Ruuskanen et al., 2013). However, RMR and field metabolic rate did not differ between chicks hatched from T- or oil-injected eggs of Black-headed gulls *Larus melanocephalus* (Eising et al., 2003).

Previous work with house sparrows in our laboratory has shown that the injection of T into the yolk of freshly laid eggs (the same dose was used in the present study) influences agonistic behavior of both sexes in non-reproductive and reproductive contexts (Strasser and Schwabl, 2004; Partecke and Schwabl, 2008) and sexual behavior of males (Partecke and Schwabl, 2008). We also showed previously that, in a common-garden aviary setting, *in ovo* T exposure increased the survival of adult females but not males, particularly during the reproductive phase (Schwabl et al., 2012). These persistent, pleiotropic, and sex-specific effects of embryonic exposure to T on adult behavior and mortality could be a consequence of developmental programming of the HPA axis and/or metabolic rate. To address this hypothesis we measured baseline and stress-induced Cort levels and resting metabolic rate (RMR) of mature male and female house sparrows that had been

exposed to a dose of testosterone (T) or vehicle *in ovo*. To assess potential effects of life history stage (season) on the expression of developmental hormonal modifications, we investigated HPA activity and RMR during two stages: first, during the non-reproductive phase (on short days, before photostimulation) and then during the reproductive phase (on longer days, after photostimulation); at these time points the birds were approximately 8, respectively 12 months old.

## MATERIALS AND METHODS

### *Ethical Statement*

All experimental procedures were approved by the Washington State University Animal Care and Use Committee as were the housing facilities.

### *General Field Procedures*

We monitored nest boxes hung in cattle barns nearby Pullman, WA (46.7º N, 117.2º W) daily for new house sparrow eggs. Each freshly laid egg of a clutch was labeled with a non-toxic marker to identify laying order, replaced with a wooden dummy egg, brought into the lab, and stored at room temperature until a clutch was complete. The third egg of each clutch was immediately frozen at -20º C for later measurement of the naturally occurring yolk T concentration (in each clutch).

### *Egg injections*

Following previously published protocol (Schwabl, 1996a; Strasser and Schwabl, 2004), all eggs of a clutch, except for the third egg (see above), were injected into the yolk either with 200 ng of T in 5 µL of sesame oil or with 5 µL of sesame oil only within 24 h of the last egg of a clutch being laid. This dose is equivalent to the highest doses of maternal T measured in clutches of the house sparrow in our study populations (Egbert et al., 2013). Of the three androgens (testosterone (T), androstenedione ($A_4$), and 5a-dihydrotestosterone (DHT)) measured in yolks of house sparrow eggs, T is the one with the highest concentrations, its concentrations varying greatly between clutches (Egbert et al., 2013). An injected dose of 200 ng of T produced various long-

lasting effects in previous studies (Partecke and Schwabl, 2008; Strasser and Schwabl, 2004; Schwabl et al., 2012). We alternated T and oil injections between clutches to control for seasonal changes in egg quality or other variables that could influence offspring phenotype. After injection, the hole in the eggshell was sealed with OpSite® transparent wound dressing (Smith & Nephew Medical, Ohio). The eggs of each clutch were then returned together into their original nest for incubation and rearing of nestlings until the age of 8–9 days. Due to this design we were unable to match nestlings to egg laying order. A total of 17 clutches were injected with T, 20 clutches received oil injections. Laying date and clutch size did not differ significantly between treatment groups (laying date C-clutches: median May 9, range 69 days; T-clutches: median April 30, range 75 days, Mann-Whitney U = 307.5, n(C-clutches) = 20, n(T-clutches) = 17, p = 0.93; clutch size: C-clutches: mean 4.46, s.e.m. 1.7 eggs; T-clutches: mean 4.2, s.e m. 1.3 eggs, $F_{1,35}$ = 1.05, p = 0.31). The laying dates cover the first half of the normal breeding season of our house sparrow population which lasts, on average, from late March to late July (unpublished data). Injections reduced hatching success compared to untreated eggs (Stewart and Westneat, 2012), but T- and C-groups did not differ in hatching success (C-eggs: 56%, T-eggs: 45%; F = 2.77, df = 1, p = 0.11). Brood size at hatching and sex ratio did not differ (C-clutches: mean 2.0, s.e.m. 2.6 nestlings; sex ratio 0.60 m/f; T-clutches: mean 2.7, s.e.m. 1.9 nestlings, sex ratio 0.53 m/f; $F_{1,28}$ = 1.09, p = 0.31). Endogenous yolk T concentrations of clutches (measured in 3$^{rd}$ eggs) were similar in T- and C-clutches. T-clutches: mean = 41.1 pg/mg, s.d.m. = 18.2 pg/mg, range 17.7 – 71.2 pg/mg, n = 16; C-clutches: mean = 34.3 pg/mg, s.d.m. = 15.9 pg/mg, range 12.9 – 79.7 pg/mg, n = 14; $F_{1,29}$ = 1.17, p = 0.289).

***Housing***

We collected the nestlings of 15 successfully hatched T- respectively 15 C-clutches from their nests at an age of 8–9 days, banded them with a numbered aluminum ring and a color band for individual identification, and hand-reared them in the laboratory with Kaytee Exact Hand-Feeding Formula (Kaytee Products, Chilton, WI). Nestlings were first housed by brood in a nest box (12.5 x 15 x 12.5 cm) and after fledging in a

cage (45 x 22 x 25 cm). When birds were feeding independently at an age of 32.7 ± 1.05 days (mean ± s.e.m.), they were moved to individual cages (45 x 22 x 25 cm) in three adjacent indoor rooms and supplied *ad libitum* with a mixture of commercial wild bird seeds and chick starter pellets and water. Birds experienced a simulated natural photoperiod of Pullman (46°43'N, 117°10' W) and temperature conditions varying between 20 - 28 ºC. Individuals of both treatment groups and sexes were intermixed in rooms and able to hear and see each other.

***Gonad size***

To assess reproductive state we measured gonad size on March 10 and 11 (approx. photoperiod 11.5 L 12.5 D) by laparotomy under local anesthesia using lidocaine cream (Wingfield & Farner, 1976). Incisions were treated with Actihaemyl gel (SolcoSwitzerland) and sealed with Histoacryl (Braun). We measured gonad size again between May 12 and June 8 (approx. photoperiod 15L 9D) when the experiment was terminated and birds were sacrificed. Using calipers, we took the width of the left testis to the nearest 0.1 mm in males and the diameter of the largest ovarian follicle to the nearest 0.1 mm in females. We did not measure gonad size in January because during this time birds were kept on short days (approx. photoperiod 8.5 L 15.5 D) that do no stimulate gonadal growth in the house sparrow (Donham et al., 1982). Egg laying starts in our field populations on average in late March.

***Stress protocol***

In January (4-11, approx. photoperiod 8.5 L 15.5 D) and April (4-11, approx. photoperiod 13 L 11 D) we determined baseline and stress-induced Cort levels by applying a standard capture and handling protocol to all individuals. On each sampling day investigators entered each of the three bird rooms to take blood samples at 0900 hours and chose an equal number of T- and C-treated house sparrows of both sexes; depending on the number of available personnel 2 or 3 birds per room were bled at the same time on a given day. Immediately after capturing an individual bird from its cage, an investigator obtained an initial blood sample (50 µL) by puncturing a brachial wing vein with a 25-gauge needle and collecting blood into

heparinized microcapillary tubes. The initial blood samples, referred to as 0 min after capture, were collected within approx. 3 min after entering the bird room (mean delay during January sampling = 50 sec, range 9 – 186 sec; mean delay during April sampling = 60 sec; range 11 – 114 sec). Samples obtained after such short delay after capture approximate baseline, non-stress Cort levels (e.g. Romero and Reed, 2005; Schwabl et al. 2016; Small et al., 2017). Indeed, the initial Cort levels (labeled as time 0) were not related to sampling delay (both January and April sampling $p > 0.05$). After collecting the initial sample, each bird was individually kept in a cloth holding bag for a 60-min period of restraint, with subsequent blood samples taken at 15, 30, and 60 min after entering the bird room to obtain stress-induced Cort levels. Blood samples were kept on ice for up to 2 h and then centrifuged at 9000 rpm for 10 min. Plasma was removed and frozen at -20°C until hormone analysis. Housing room, bleeder identity, and bleeding order in and among rooms had no effect on initial Cort levels (all $p > 0.05$).

***Resting metabolic rate***

We performed respirometry in January (22 – 31, approx. photoperiod 9 L 15 D) and again in April (18 – 27, approx. photoperiod 14 L 10 D), when gonads were undeveloped, respectively growing. Resting metabolic rate (RMR) was calculated by measuring $O_2$ consumption in an open flow, push-through respirometry system (Withers, 1977). Each afternoon at 15:00 h, a random set of seven birds was transported in cloth bags from their living cages to an adjacent climate-controlled chamber, where all measurements took place under simulated natural day length conditions. Metabolic rate was measured continuously from 23:00 to 09:00 of the following day, using 3.8 l plastic jars with screw-on lids as metabolic chambers, at 25 ± 2°C, within the thermoneutral zone (McNab, 2012). We monitored ambient temperature (25 ± 1.5 °C) in the base-lining (control) respirometry chamber with an electronic thermometer (Radio Shack Inc.). Treatment groups and sexes were equally distributed within and across measurement sessions. During metabolic measurements, birds had free access to food and water. To quantify $O_2$ consumption we used ultra-low permeability Tygon tubing with an internal diameter of 0.32 mm.

Room air was pumped through both a Drierite column (to remove water vapor) and a $CO_2$ scrubber (Ascarite) before passing into a gas mass flow controller (Cole-Palmer Inc.). Flow rate out of the gas mass flow controller was set to 4.1 L/ min. The air stream was then split by a manifold chamber (Sable Systems). Air from the chambers was sent through a TR-RM8 respirometer multiplexer (Sable Systems) and a second Drierite column before being sub-sampled at a rate of 150 mL/min before entering the $CO_2$ analyzer (CA 10a, Sable Systems). The $CO_2$ analyzer was calibrated each morning with air from a known gas mixture (5% $CO_2$). The sample air was then scrubbed of $CO_2$ in a second Ascarite column before moving to the $O_2$ analyzer (FC 10a, Sable Systems). Room air that had been scrubbed of water vapor and $CO_2$ was pumped through an $O_2$ analyzer as a control; the difference between the two values (sample air and control air) was recorded.

During each trial, we programmed our automated multiplexed respirometry system to measure $O_2$ consumption and $CO_2$ production for each bird at 1 s intervals for 10 min per chamber, then switch to the next chamber in series. After the completion of each series of seven 10-min sampling periods, we measured the same gas concentrations in an empty chamber to obtain baseline gas levels passing through experimental chambers. We then began a new series of seven 10-min sampling periods, repeating this process throughout the night. By the end of the trial each morning, we had acquired at least six 10-min sampling intervals per bird for the previous night. To determine RMR for each bird during the night, we identified the 5-min period of lowest $O_2$ consumption per night for each bird. Birds were weighed before and after respirometry and the mean of these measurements was used to obtain mass-specific RMR (mRMR) expressed as ml $O_2$/(min x g) (Fig. 3).

***Corticosterone assay***

We measured plasma Cort concentration by radioimmunoassay (Wingfield et al., 1992). Antiserum was purchased from Esoterix Endocrinology (Calabasas Hills, USA). Standard steroids were obtained from Sigma-Aldrich (Munich, Germany) and $^3$H-labeled corticosterone from Perkin Elmer (Rodgau, Germany). All samples of an individual including both seasons (January and April) and paired samples from T- and C-birds were assigned to the same purification run (N=5) and assay (N=6). Plasma

was equilibrated with a small dose of tritiated corticosterone (2000 cpm) to measure subsequent recovery. Corticosterone was extracted with redistilled dichloromethane (4 ml) following an established protocol (Hall et al., 1987). Each sample was then assayed in duplicate. Extraction recovery was 76% ± 0.4% (mean ± SE). Intra- and inter-assay variation (N = 6) varied between 6–10%. The least detectable plasma concentration (calculated for mean recovery rate and mean plasma volume) was 72.4 pg/mL.

***Yolk T assay***

As a measure of the naturally occurring inter-clutch variation in yolk T concentration we quantified yolk T concentrations of the third egg of each clutch using separation protocols and radioimmunoassay as described (Schwabl, 1993). Weighed amounts (approx. 200 mg) of defrosted and homogenized yolk were diluted with 200 µl distilled water. After adding 20 µl tritiated $A_4$, 5α-DHT, T, and 17b-estradiol ($E_2$) to each sample for calculation of recoveries, samples were extracted twice with 4 ml petroleum ether/diethyl ether (30/70%), followed by precipitation with 90% ethanol to remove neutral lipids. Subsequently, the hormones were separated on diatomaceous earth chromatography columns. Briefly, samples were reconstituted in 10% ethyl acetate in 2,2,4-trimethylpentane and then transferred to the columns. $A_4$ was eluted with a concentration of 2% ethyl acetate in 2,3-trimethyl-pentane, 5α-DHT with 10% ethyl acetate, T with 20% ethyl acetate, and $E_2$ with 40 %. T concentrations were measured in double competitive-binding radioimmuno-assays (RIA) with $^3$H-labelled T (NET 553) and was obtained from PerkinElmer Life and Analytical Sciences. T antibody was T 3003 (Wien Laboratories). Average T recovery was 68.4%. Mean intra-assay variation was 8.4%. Detection limits (pg/mg yolk) of the steroid RIA was 0.05 pg/mg.

***Data analyses***

Statistical analyses were performed in SPSS 25/26 using linear mixed effect models (LMEs) fitted by restricted maximum likelihood. To meet the assumptions of LME, response variables were transformed for normality, when needed; model residuals were normally distributed.

Testes and ovary size were analyzed using LME with reproductive state (March or May-June) and egg treatment as fixed factors.

We ln-transformed Cort concentrations before LME to analyze basal and stress-induced levels. Cort levels were analyzed separately for the non-reproductive (January) and reproductive (April) state to reduce the number of complex multiway interaction that are difficult to interpret. In these analyses time (0, 15, 30, 60 min of handling stress) was a repeated measure effect and sex and treatment (T or C injection) were fixed effects; the brood specific variables of hatch date (Julian date) and ln-transformed yolk T concentrations (measured in the third egg of each clutch to assess inter-female yolk T variation) were used as covariates.

Body mass was analyzed by LME with reproductive state (non-reproductive (January) versus reproductive (April)) as repeated measure effect, sex, and egg treatment as fixed factors, and yolk T concentration (ln-transformed) and Julian date (brood hatch date) as covariates. Yolk T concentrations and hatch date had similar means and ranges in T- and C-clutches.

RMR was corrected for body mass to generate mass-specific RMR (mRMR) which was analyzed by LME with reproductive state (non-reproductive (January) versus reproductive (April)) as repeated measure effect. Fixed effects were sex and treatment; the brood-specific variables (hatch date and ln yolk T) were included as covariates. To assess effects of Cort levels on mRMR we included basal and integrated Cort concentrations (total Cort levels under the curve of the stress response) as covariates.

All models included nest ID as random factor to account for multiple siblings in broods (see injections above). We used backward elimination of least significant terms and applied Aikaike's Information Criterion to evaluate and select best models (Aikaike, 1973). We report statistics for fixed effects of the best model. When reporting non-significant effects of co-variates such as hatch date and clutch yolk T we used output from initial models that included all variables.

## RESULTS

### *Gonadal growth (Fig. 1)*

As expected, gonads were small and undeveloped in March and increased in size between March and May/June (reproductive state: testes $F_{1,34}$ = 327.50, $p$ < 0.001, ovaries $F_{1,24}$ = 41.270, $p$ < 0.001; Fig. 1*).* Testes growth was not affected by egg treatment (state*treatment: $F_{1,34}$ = 1.920, $p$ = 0.17), although males from T-treated eggs tended to have somewhat larger testes than controls (treatment: $F_{1,34}$ = 3.171, $p$ = 0.08). T treatment did not influence ovarian follicle size and growth (treatment: $F_{1,24}$ = 0.626, $p$ = 0.44, state*treatment: $F_{1,24}$ = 0.390, $p$ = 0.53).

### *Baseline corticosterone and stress response profile (Fig. 2):*

Cort levels at time "0 min" (see Methods for exact times) were not related to sampling delay (up to 186 sec, p > 0.05, also see Methods) and thus can be assumed to represent baseline Cort concentrations. Baseline Cort levels were higher after the onset of gonad growth (April) than before (January) ($F_{1,94}$ = 28.444, $p$ < 0.001). Sex and treatment interacted to affect baseline Cort levels (sex*treatment: $F_{1,101}$ = 5.460, $p$ = 0.02), with slightly higher levels in T- than C-males (C: mean = 1.46 ng/ ml, s.e.m. = 0.11; T: mean = 1.79 ng/ml, s.e.m. = 0.15), but no difference in females (C: mean = 1.66 ng/ml, s.e.m. = 0.15; T: mean = 1.67 ng/ml; s.e.m. = 0.17). Baseline Cort levels were neither related to hatch date ($F_{1, 46}$ = 0.46; p = 0.831) nor to endogenous yolk T concentrations ($F_{1, 37}$ = 1.93; p = 0.173)**.**

During the non-reproductive phase (January) neither sex nor treatment (nor the initially included 3-way and 2-way interactions) had effects on plasma Cort levels during the stress test (sex: $F_{1,149}$= 1.556, p = 0.214; treatment: $F_{1,149}$ = 1.084, p = 0.299); stress time had a strong effect ($F_{3,127}$ = 349.80, p < 0.001). In contrast, during the reproductive phase (April) stress time ($F_{3,122}$ = 178.87; p < 0.001) and the 3-way interaction of stress time, sex, and treatment ($F_{4,61}$ = 2.69; p = 0.039) affected Cort levels. Sex and treatment alone as well as their interactions with stress time had no effect (all p > 0.27). When the analyses were conducted separately for each sex, treatment had a main effect on Cort levels in females during the reproductive phase ($F_{1,100}$ = 6.46; p = 0.013), but not the non-reproductive phase ($F_{1,114}$ = 0.037; p =

0.847). There was no main effect of treatment in either phase in males (both p > 0.256). Note the prolonged stress-induced Cort secretion in females from T-treated eggs compared to females from control eggs in April, but not in January: while the Cort levels of control females ceased rising and started to decrease by 15 min they continued to rise until 30 min in T-treated females (Fig. 2D). This profile resembles that observed in both sexes (regardless of treatment) before the onset of gonad growth (January). Post-hoc analyses restricted to females during the reproductive phase (April) reveal significantly higher Cort levels at 30 min in females from T-treated compared to those from control eggs ($F_{1,29}$ = 4.561; p = 0.043). Hatch date ($F_{1,40}$ = *0.27; p = 0.606*) and endogenous yolk T concentration ($F_{1, 41}$ = *1.39; p = 0.245*) had no effect on stress-induced Cort levels.

In summary, yolk T injections resulted in overall slightly elevated baseline Cort levels in males but not females and protracted stress-induced Cort secretion in females but not males, but only after onset of gonadal development.

***Body mass***

Body mass was significantly influenced by reproductive state ($F_{1,93}$ = 16.319; p < 0.001) and the interaction of reproductive state and sex ($F_{1,93}$ = 4.524; p = 0.036), but it was not affected by treatment ($F_{1,38}$ = 1.327; p = 0.256) and only marginally by sex ($F_{1,122}$ = 3.645; p = 0.059). It was related to hatch date ($F_{1,38}$ = 4.583; p = 0.039), with individuals of broods hatched later in the season being heavier as adults than those hatched earlier. Mass was not related to endogenous clutch yolk T concentration ($F_{1, 28}$ = 0.212; p = 0.649).

***Mass-specific resting metabolic rate (mRMR) (Fig. 3)***

Treatment, sex, and reproductive state had no main effects on mRMR (treatment: $F_{1,384}$ = 1.755; p = 0.194; sex: $F_{1,127}$ = 1.415; p = 0.236; reproductive state: $F_{1,102}$ = 2.988; p = 0.087). Sex and reproductive state interacted to influence mRMR ($F_{1,102}$ = 4.74; p = 0.032), reflecting an increase in mRMR by about 5% in males but not in females after onset of gonad growth (April) compared to when gonads were undeveloped in January. Two- and three-way interactions were not significant (all p >

0.10). The covariates hatch date, yolk T, and baseline Cort made no significant contribution to variation in mRMR (hatch date: $F_{1, 31} = 0.011$; p = 0.917; yolk T: $F_{1, 28} = 1.72$; p = 0.20; baseline Cort; $F_{1,109.} = 0.006$; p = 0.941). The final model included a significant effect of total integrated Cort during stress (area under the curve from time 0 min to time 60 min in the stress test) ($F_{1,118} = 6.030$; p = 0.016), suggesting that mRMR and overall stress-induced Cort production are positively correlated with each other.

## DISCUSSION

The main results of this experiment summarize as follows. *In ovo* T treatment programmed sex- and state-specific modifications of HPA activity: Males exposed to T in the egg showed slightly higher baseline Cort levels but a similar stress response compared to control males; females exposed to T in the egg, in contrast, showed similar baseline Cort levels compared to control females, but an exaggerated secretion of Cort during stress; however, this effect in females became only evident after the onset of ovarian growth. Mass-specific metabolic rate (mRMR) was not modified by yolk T injections but related to overall stress Cort secretion. These results do not support the hypothesis that previously reported pleiotropic effects of yolk T on adult behavior result from long term modification of basal metabolic rate. Programming of the HPA axis (baseline secretion in males and stress-induced secretion in reproductive females) by yolk T, on the other hand, may be linked to some of the observed behavioral effects in adult males and females and differences in survival rate of adult females.

Dynamic changes in circulating glucocorticoids of vertebrates regulate metabolic support of "routine" day-to-day performance as well as physiological and behavioral responses to unpredicted perturbation of homeostasis by stressors (Sapolsky et al., 2000; Wingfield et al., 1998). Low, baseline circulating levels act via the high affinity GR I receptor system to regulate intermediary metabolism, while high, stress-induced Cort levels act via the low affinity GR II receptor system to induce transient physiological and behavioral responses to cope with stressors (de Kloet et

al., 1990; Lattin and Romero, 2015; Romero, 2004). In males, we found slightly increased baseline Cort levels in T-treated individuals, while in females the stress-induced Cort secretion profile, but not baseline Cort was affected by *in ovo* T treatment. We have no explanation for the mechanism by which developmental T exposure might program increased baseline Cort secretion in males, but a likely explanation for the changed Cort stress secretion profile of females may be modification of the negative feedback system for down-regulation of stress-induced Cort (Liebl et al., 2013). There is good evidence in rodents and birds that developmental programming of the HPA axis results in changes of adult stress glucocorticoid attenuation via the permanent down/up-regulation of brain GR II receptors (e.g. Banerjee et al., 2012; Weaver et al., 2004); and this can occur in a sex-specific manner (McCarthy et al., 2009; McCarthy and Nugent, 2013; Menger et al., 2010). The absence of a difference in the stress response profile between T- and control females before the onset of ovarian growth suggests that photostimulation and/or activation of the hypothalamus-pituitary-ovary axis are required for the expression of this maternal effect in a state-dependent manner; we did, however, not detect differences in ovarian follicle size (this study) or circulating levels of ovarian steroids (progesterone, testosterone, 5α-dihydrotestosterone, and 17ß-estradiol) between adult females from T-treated and control eggs (Partecke and Schwabl, 2008) that could be related to the modified stress response.

Regardless of the exact nature of the underlying mechanisms, elevated baseline Cort in males and prolonged secretion of Cort during stress episodes in females could impact performance (Patterson et al., 2014; Vitousek et al., 2014). This programming might cause some of the behavioral effects of yolk T that we reported previously (Partecke and Schwabl, 2008; Strasser and Schwabl 2004); and, one could also speculate that the modified stress Cort secretion profile of females during the reproductive phase is related to a reduced mortality risk of T-treated compared to control females that we found in a previous aviary study (Schwabl et al., 2012). Free-living house sparrows caught at their nests exhibited substantial individual variation of Cort levels in response to 30 min of standardized capture and handling stress (Lendvai et al. 2007); and, in females, 30 min stress-induced Cort levels were

negatively related to inquisitive behavior in response to novelty, a component of coping style (Lendvai et al. 2011).

Our *in ovo* T treatment did not affect adult mass-specific RMR. This outcome differs from experimental studies of two other passerine bird species, domesticated zebra finch (*Taeniopygia guttata*) and wild pied flycatcher (*Ficedula hypoleuca*) that both reported enhanced adult metabolic rate by *in ovo* androgen treatment (T in the zebra finch (Nilsson et al., 2011); T plus androstenedione in the pied flycatcher (Ruskaanen et al., 2013)). A third study conducted with the non-passerine Black-headed gull (*Larus ridibundus*) did, however, not detect any effects of egg T treatment on RMR and field metabolic rate of nestlings (Eising et al., 2003). It remains to be understood why yolk androgen manipulation influences RMR in some species but not in others. Nevertheless, the absence of an effect of T treatment on mRMR in our study indicates that the observed effects of yolk T on adult behavior (Partecke and Schwabl, 2008; Strasser and Schwabl, 2004) are not a consequence of modified metabolic rate (Biro and Stamps, 2010; Mathot and Dingemanse, 2015). Similarly, the different adult mortality rates of T-injected and control females (Schwabl et al., 2012) do not appear to be associated with developmentally programmed differences in metabolic rate and their potential consequences for health and disease.

Metabolic rate has been found to co-vary with baseline and stress-induced glucocorticoid levels at the intra-specific level (Jimeno et al., 2017; Welcker et al., 2015; but see Buehler et al., 2012; Welcker et al., 2009). We found a positive correlation of mRMR with the integrated overall Cort secretion during a stress episode, but not with baseline Cort levels. Although we did not measure Cort and mRMR at exactly the same times, it is possible that our respirometry procedure which included confinement of the bird in a small chamber represented a stressful situation which triggered a Cort stress secretion response similar to that measured in the stress protocol. In this case, our results might suggest that developmental T exposure may indirectly influence RMR via modification of glucocorticoid secretion during stress.

Our present and previous research (Partecke and Schwabl, 2008; Strasser and Schwabl, 2004; Schwabl et al., 2012) with the house sparrow shows that variation in developmental exposure to a single hormone such as T influences multiple adult traits, from morphology, to behavior, to the stress response. These effects can be sex-specific and even differ between the sexes in the specific components that are affected, as shown here for the HPA axis where basal Cort levels were affected in males but not females, and stress Cort levels in females but not in males. In addition, we show here for the HPA stress response that the expression of maternal programming depends on life history-stage and context (reproductive versus non-reproductive). This dependency on sex and stage adds another layer of complexity to hormone-mediated maternal effects that needs to be considered when assessing mechanisms and functions. It further supports the perspective that maternal steroids do not simply interfere or interact with the hormonal processes of normal sexual differentiation (Carere and Balthazart, 2007). Rather they may act through epigenetic pathways and mechanisms such as DNA methylation and histone modification (Forger 2016), even before organogenesis and sexual differentiation (Kumar et al., 2018; Schwabl and Groothuis 2010), to cause permanent modifications of traits.

In conclusion, we show that maternal androgens (T) can program components of the HPA axis while research with other species, focusing on a direct linkage of glucocorticoid stress hormones themselves across generations, has shown that *in ovo* Cort (elevated as a consequence of enhanced maternal HPA activity and stress) can influence offspring HPA activity (Hausmann et al., 2012; Hayward and Wingfield, 2004; Hayward et al., 2006; Marasco et al., 2012; Nesan and Vijayan, 2016; Thayer et al., 2018; Zimmer et al., 2017). Apparently, the maternal organism is linked to the offspring via multiple non-genomic signals including various hormones that converge to modify the function of certain traits and systems such as the HPA axis. This redundancy and complexity emphasizes the significance of transmission of non-genomic maternal information and input for development but also complicates progress towards a comprehensive understanding of the epigenetic developmental processes by which diverse hormonally-mediated and other maternal effects act and are integrated to shape offspring phenotype.

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**Data Availability:**

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing Interests:**

The authors have no competing interests to report.

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## Figures:

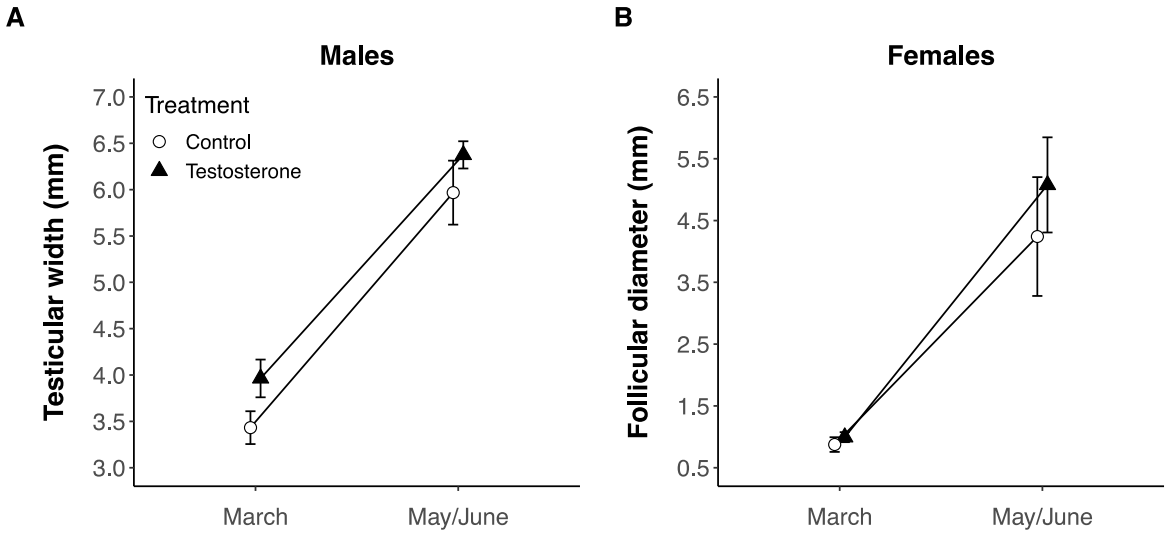


**Figure 1.** Testis width (A) and largest ovarian follicle diameter (B) (mean ± 1 SEM) in March (controls: 17 males, 12 females; T-treated: 18 males, 17 females) and May/June (controls: 17 males, 11 females; T-treated: 18 males, 13 females) of house sparrows hatched from T-treated and control eggs.

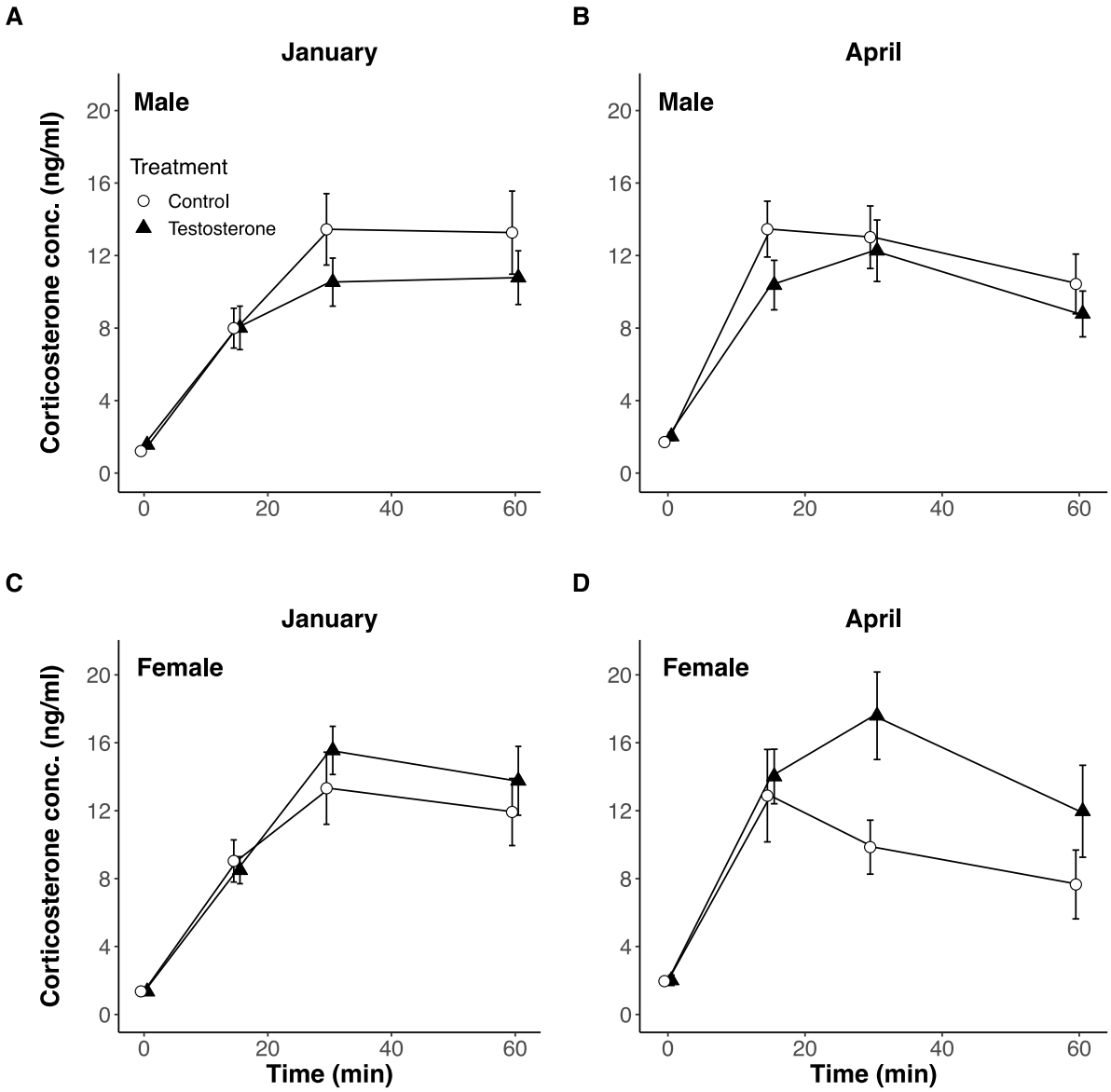


**Figure 2.** Plasma concentrations (mean ± 1 SEM) of baseline (time = 0 min) and stress-induced corticosterone during a 60 min standard capture and handling stress protocol of male (A, B) and female (C, D) house sparrows hatched from T-treated and control eggs in January (non-reproductive phase, A and C) and April (reproductive phase, B and D). January: controls: 18 males, 12 females; T-treated: 19 males, 17 females; April: 18 males, 12 females; T-treated: 17 males, 17 females.

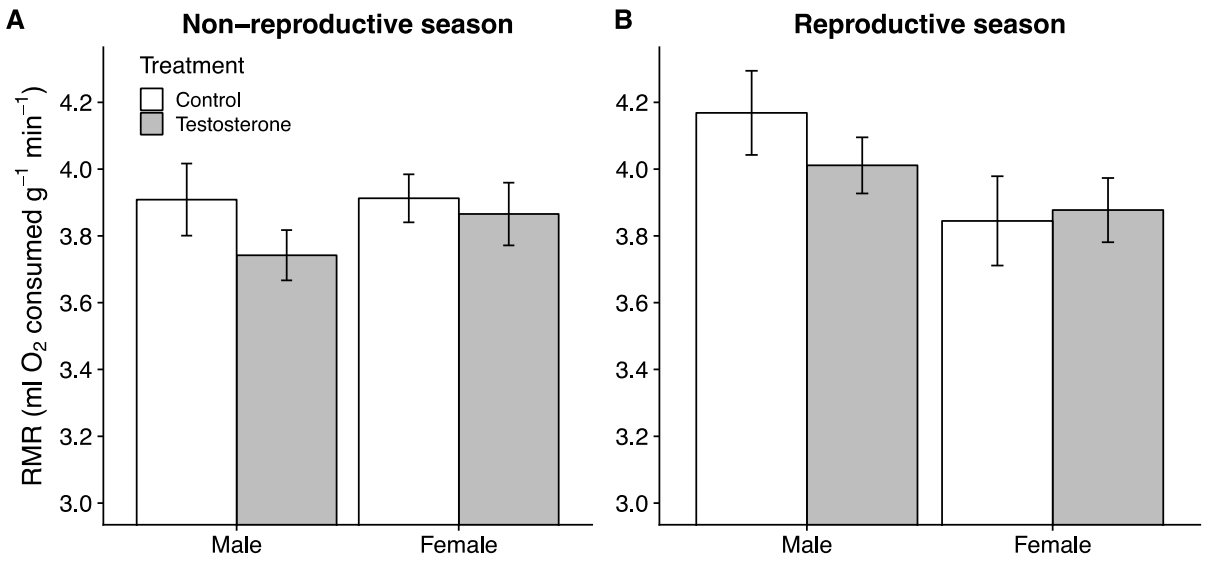


**Figure 3.** Mass-specific resting metabolic rate (mRMR, mean ± 1 SEM) during the non-reproductive (January, A) and reproductive (April, B) phase of male and female house sparrows hatched from T-treated and control eggs. January: controls: 18 males, 12 females; T-treated: 19 males, 17 females; April: controls: 18 males; 12 females; T-treated: 19 males; 17 females.