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RESEARCH ARTICLE

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 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 such as adrenocortical activity or metabolic rate. We investigated whether experimentally increased *in ovo* exposure to testosterone influenced hypothalamus–pituitary–adrenal function, i.e. baseline and stress-induced corticosterone 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 testosterone exposure on adult agonistic and sexual behavior and survival. Here we report that baseline corticosterone levels and the stress secretion profile of corticosterone are modified by *in ovo* testosterone in a sex-specific and life history stage-dependent manner. Compared with controls, males from testosterone-treated eggs had higher baseline corticosterone levels, whereas females from testosterone-treated eggs showed prolonged stress-induced corticosterone secretion during the reproductive but not the non-reproductive phase. Adult RMR was unaffected by *in ovo* testosterone treatment but correlated with integrated corticosterone stress secretion levels. We conclude that exposure of the embryo to testosterone programs the hypothalamus–pituitary–adrenal axis in a sex-specific manner that in females depends, in expression, on reproductive state. The modified baseline corticosterone levels in males and stress-induced corticosterone levels in females may explain some of the long-lasting effects of maternal testosterone in the egg on behavior and could be linked to previously observed reduced mortality of testosterone-treated females.

KEY WORDS: Developmental programming, Hormone, Maternal effect, Pleiotropy, Sex difference, Stress response

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: Groothuis et al., 2019; Podmokla et al., 2018; Schwabl, 1993; Schwabl and Groothuis, 2010; 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; Ruuskanen 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 and 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; Adkins-Regan et al., 2013; Carere and Balthazart, 2007) they probably 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 trade-off, or it may be the consequence of modification of fundamental systemic processes that affect the function of other functions (Groothuis and Schwabl, 2008). Here we investigated the effects of developmental exposure to testosterone on two fundamental organismal processes that are known to affect and modify many other systems, functions and traits: the hypothalamus–pituitary–adrenal (HPA) axis (Crino et al., 2016; 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; Holtmann et al., 2017; Mathot and Dingemanse, 2015; Mathot et al., 2015).

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; Romero, 2002; 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

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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. Astheimer et al., 1994, 1995; Baugh et al., 2012; Gómez et al., 1998; Gormally and Romero, 2018; Novais et al., 2017; Sapolsky et al., 1986; Schmidt et al., 2012; Schwabl, 1995; Small and Schoech, 2015). 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, 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 corticosterone levels than those from control eggs (Sockman and Schwabl, 2001). Long-lasting effects of exposure to maternal androgens in the eggs on baseline corticosterone 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 have an impact on an offspring's phenotype by effects on development rate (Martin and Schwabl, 2008; Schwabl et al., 2007), 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 variations in behavior (e.g. Biro and Stamps, 2010; Careau and Garland, 2012; Holtmann et al., 2017; Killen et al., 2013; Mathot et al., 2015) and impacts on aging, mortality and fitness (Burton et al., 2011; Harman, 1956). Experimentally elevated *in ovo* androgen exposure increased RMR in nestling (testosterone 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 testosterone plus androstenedione treatment; Ruuskanen et al., 2013). However, RMR and field metabolic rate did not differ between chicks hatched from testosterone- or oil-injected eggs of black-headed gulls (*Larus ridibundus*) (Eising et al., 2003).

Previous work with house sparrows in our laboratory has shown that the injection of testosterone 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 (Partecke and Schwabl, 2008; Strasser and Schwabl, 2004) and sexual behavior of males (Partecke and Schwabl, 2008). We also showed previously that, in a common garden aviary setting, *in ovo* testosterone 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 testosterone 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 corticosterone levels and RMR of mature male and female house sparrows that had been exposed to a dose of testosterone 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 and 12 months old, respectively.

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 near Pullman, WA, USA (46.7°N, 117.2°W), daily for new house sparrow [*Passer domesticus* (Linnaeus 1758)] 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 laboratory, and stored at room temperature until a clutch was complete. The third egg of each clutch was immediately frozen at −20°C for later measurement of the naturally occurring yolk testosterone concentration (in each clutch).

Egg injections

Following a previously published protocol (Schwabl, 1996; 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 testosterone in 5 µl of sesame oil or with 5 µl of sesame oil only (control, C) within 24 h of the last egg of a clutch being laid. This dose is equivalent to the highest doses of maternal testosterone measured in clutches of the house sparrow in our study populations (Egbert et al., 2013). Of the three androgens [testosterone, androstenedione and 5α-dihydrotestosterone (DHT)] measured in yolks of house sparrow eggs, testosterone is the one with the highest concentrations; its concentrations vary greatly between clutches (Egbert et al., 2013). An injected dose of 200 ng of testosterone produced various long-lasting effects in previous studies (Partecke and Schwabl, 2008; Schwabl et al., 2012; Strasser and Schwabl, 2004). We alternated testosterone 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, OH, USA). 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 testosterone (T-clutches), and 20 clutches received oil injections (C-clutches). Laying date and clutch size did not differ significantly between treatment groups [laying date of C-clutches: median 9 May, range 69 days; T-clutches: median 30 April, range 75 days, Mann–Whitney U=307.5, $N_{\text{C-clutch}}$=20, $N_{\text{T-clutch}}$=17, P=0.93; clutch size: C-clutches, 4.46±1.7 eggs (mean±s.e.m.); T-clutches, 4.2±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 (H.S., unpublished data). Injections reduced hatching success compared with untreated eggs (Stewart and Westneat, 2013), but testosterone and control groups did not differ in hatching success (C-eggs: 56%, T-eggs: 45%; F=2.77, d.f.=1, P=0.11). Brood size at hatching and sex ratio did not differ [C-clutches: 2.0±2.6 nestlings (mean±s.e.m.); sex ratio 0.60 males

to females; T-clutches: mean 2.7±1.9 nestlings, sex ratio 0.53 males to females; $F_{1,28}$=1.09, P=0.31]. Endogenous yolk testosterone concentrations of clutches (measured in third eggs) were similar in T- and C-clutches; T-clutches: 41.1±18.2 pg mg^{-1} (mean±s.d.), range 17.7–71.2 pg mg^{-1}, N=16; C-clutches: 34.3±15.9 pg mg^{-1}, range 12.9–79.7 pg mg^{-1}, N=14; $F_{1,29}$=1.17, P=0.289).

Housing

We collected the nestlings of 15 successfully hatched T-clutches and 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, USA). Nestlings were first housed by brood in a nest box (12.5×15×12.5 cm) and after fledging in a cage (45×22×25 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 (45×22×25 cm) in three adjacent indoor rooms and supplied *ad libitum* with a mixture of commercial wild bird seed 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 and 28°C. Individuals of both treatment groups and sexes were intermixed in rooms and were able to hear and see each other.

Gonad size

To assess reproductive state we measured gonad size on 10 and 11 March (approximate photoperiod 11.5 h:12.5 h light:dark) by laparotomy under local anesthesia using lidocaine cream (Wingfield and Farner, 1975). Incisions were treated with Actihaemyl gel (Solco, Basel, Switzerland) and sealed with Histoacryl (Braun). We measured gonad size again between 12 May and 8 June (approximate photoperiod 15 h:9 h light:dark) when the experiment was terminated and birds were euthanized by overdose of sodium pentobarbital. 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 (approximate photoperiod 8.5 h:15.5 h light:dark) that do not stimulate gonadal growth in the house sparrow (Donham et al., 1982). Egg laying started in our field populations on average in late March.

Stress protocol

In January (4–11, approximate photoperiod 8.5 h:15.5 h light:dark) and April (4–11, approximate photoperiod 13 h:11 h light:dark), we determined baseline and stress-induced corticosterone 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 09:00 h and chose an equal number of testosterone- and control-treated house sparrows of both sexes; depending on the number of available personnel, two or three 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 approximately 3 min after entering the bird room (mean delay during January sampling was 50 s, range 9–186 s; mean delay during April sampling was 60 s, range 11–114 s). Samples obtained after such a short delay after capture approximate baseline, non-stress corticosterone levels (e.g. Romero and Reed, 2005; Schwabl et al., 2016; Small et al., 2017). Indeed, the initial corticosterone 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 corticosterone levels. Blood samples were kept on ice for up to 2 h, plasma was removed 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 corticosterone levels (all P>0.05).

Resting metabolic rate

We performed respirometry in January (22–31, approximate photoperiod 9 h:15 h light:dark) and again in April (18–27, approximate photoperiod 14 h:10 h light:dark), when gonads were undeveloped and growing. 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 h of the following day, using 3.8 liter 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 baseline (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 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^{-1}. 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^{-1} before entering the CO_2 analyser (CA 10a, Sable Systems). The CO_2 analyser 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 analyser (FC 10a, Sable Systems). Room air that had been scrubbed of water vapor and CO_2 was pumped through an O_2 analyser as a control; the difference between the two values (sample air and control air) was recorded.

During each trial, we programmed our automated multi-plexed 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 g)$^{-1}$.

Corticosterone assay

We measured plasma corticosterone concentration by radioimmunoassay (Wingfield et al., 1992). Anti-serum was purchased from Esoterix Endocrinology (Calabasas Hills, CA, 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 re-distilled 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± s.e.m.). Intra- and inter-assay variation (N=6) varied between 6 and 10%. The least detectable plasma concentration (calculated for mean recovery rate and mean plasma volume) was 72.4 pg ml^{-1}.

Yolk testosterone assay

As a measure of the naturally occurring inter-clutch variation in yolk testosterone concentration, we quantified yolk testosterone concentrations of the third egg of each clutch using separation protocols and radioimmunoassay as described previously (Schwabl, 1993). Weighed amounts (approximately 200 mg) of defrosted and homogenized yolk were diluted with 200 µl distilled water. After adding 20 µl tritiated androstenedione, 5α-DHT, testosterone and 17β-estradiol to each sample for calculation of recoveries, samples were extracted twice with 4 ml petroleum ether–diethyl ether (30% and 70%, respectively), 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. Androstenedione was eluted with a concentration of 2% ethyl acetate in 2,3-trimethyl-pentane, 5α-DHT with 10% ethyl acetate, testosterone with 20% ethyl acetate, and 17β-estradiol with 40% ethyl acetate. Testosterone concentrations were measured in double competitive-binding radioimmunoassays (RIA) with ^{3}H-labeled testosterone (NET 553) obtained from PerkinElmer Life and Analytical Sciences. Testosterone antibody was T 3003 (Wien Laboratories). Average testosterone recovery was 68.4%. Mean intra-assay variation was 8.4%. Detection limit (pg mg^{-1} yolk) of the steroid RIA was 0.05 pg mg^{-1}.

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 analysed using LME with reproductive state (March or May–June) and egg treatment as fixed factors.

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

Body mass was analysed by LME with reproductive state [non-reproductive (January) versus reproductive (April)] as a repeated-measures effect, sex and egg treatment as fixed factors, and yolk testosterone concentration (ln-transformed) and Julian date (brood hatch date) as covariates. Yolk testosterone 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 analysed by LME with reproductive state [non-reproductive (January) versus reproductive (April)] as a repeated-measures effect. Fixed effects were sex and treatment; the brood-specific variables (hatch date and ln yolk testosterone) were included as covariates. To assess effects of corticosterone levels on mRMR, we included basal and integrated corticosterone concentrations (total corticosterone levels under the curve of the stress response) as covariates.

All models included nest identity (ID) as random factor to account for multiple siblings in broods (see 'Egg injections' section above). We used backward elimination of least significant terms and applied Akaike's information criterion to evaluate and select best models (Akaike, 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 testosterone, we used output from initial models that included all variables.

RESULTS

Gonadal growth

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). Growth of the testes was not affected by egg treatment (state×treatment: $F_{1,34}$=1.920, P=0.17), although males from testosterone-treated eggs tended to have somewhat larger testes than controls (treatment: $F_{1,34}$=3.171, P=0.08). Testosterone 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

Corticosterone levels at time '0 min' (see Materials and Methods for exact times) were not related to sampling delay (up to 186 s, P>0.05, also see Materials and Methods) and thus can be assumed to represent baseline corticosterone concentrations (Fig. 2). Baseline corticosterone 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 corticosterone levels (sex×treatment: $F_{1,101}$=5.460, P=0.02), with slightly higher levels in testosterone-treated than control males (control: 1.46± 0.11 ng ml^{-1} (mean±s.e.m.); testosterone: 1.79±0.15 ng ml^{-1}), but no difference in females (control: 1.66±0.15 ng ml^{-1}; testosterone: 1.67±0.17 ng ml^{-1}). Baseline corticosterone levels were not related to hatch date ($F_{1,46}$=0.46, P=0.831) or endogenous yolk testosterone concentrations ($F_{1,37}$=1.93, P=0.173).

During the non-reproductive phase (January) neither sex nor treatment (nor the initially included three- and two-way interactions) had effects on plasma corticosterone 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 three-way interaction of stress time, sex and treatment ($F_{4,61}$=2.69, P=0.039) affected corticosterone 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

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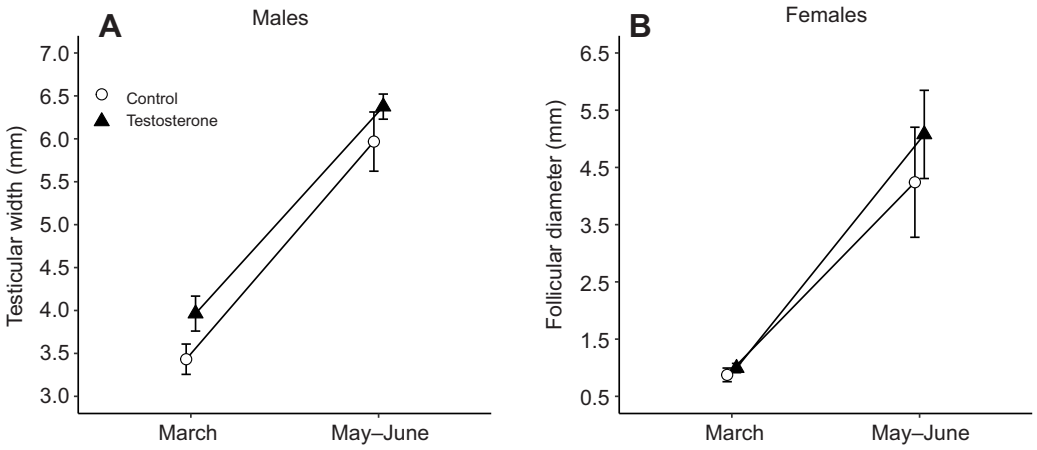

Fig. 1. Gonadal growth in *Passer domesticus*. (A) Testis width and (B) largest ovarian follicle diameter (mean±1 s.e.m.) in March (controls: 17 males, 12 females; testosterone-treated: 18 males, 17 females) and May–June (controls: 17 males, 11 females; testosterone-treated: 18 males, 13 females) of house sparrows hatched from testosterone-treated and control eggs.

for each sex, treatment had a main effect on corticosterone 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 corticosterone secretion in females from testosterone-treated eggs compared with females from control eggs in April, but not in January: while the corticosterone levels of control females ceased rising and started to decrease by 15 min they continued to rise until 30 min in testosterone-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 corticosterone levels at 30 min in females from testosterone-treated eggs compared with 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 testosterone concentration ($F_{1,41}$=1.39, P=0.245) had no effect on stress-induced corticosterone levels.

In summary, yolk testosterone injections resulted in overall slightly elevated baseline corticosterone levels in males but not females and protracted stress-induced corticosterone 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 testosterone concentration ($F_{1,28}$=0.212, P=0.649).

Mass-specific resting metabolic rate

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; Fig. 3). Sex and reproductive state interacted to influence mRMR ($F_{1,102}$=4.74, P=0.032), reflecting an increase in mRMR by approximately 5% in males but not in females after onset of gonad growth (April) compared with when gonads were undeveloped in January. Two- and three-way interactions were not significant (all P>0.10). The covariates hatch date, yolk testosterone and baseline corticosterone made no significant contribution to variation in mRMR (hatch date: $F_{1,31}$=0.011, P=0.917; yolk testosterone: $F_{1,28}$= 1.72, P=0.20; baseline corticosterone: $F_{1,109}$=0.006, P=0.941). The final model included a significant effect of total integrated corticosterone 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 corticosterone production are positively correlated with each other.

DISCUSSION

The main results of this experiment are summarized as follows. *In ovo* testosterone treatment programmed sex- and state-specific modifications of HPA activity. Males exposed to testosterone in the egg showed slightly higher baseline corticosterone levels but a similar stress response compared with control males. Females exposed to testosterone in the egg, in contrast, showed similar baseline corticosterone levels compared with control females, but an exaggerated secretion of corticosterone during stress. However, this effect in females only became evident after the onset of ovarian growth. Mass-specific metabolic rate was not modified by yolk testosterone injections but related to overall stress corticosterone secretion. These results do not support the hypothesis that previously reported pleiotropic effects of yolk testosterone 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 testosterone, however, 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 glucocorticoid GRI receptor system to regulate intermediary metabolism, while high, stress-induced corticosterone levels act via the low-affinity GRII receptor system to induce transient physiological and behavioral responses to cope with stressors (de Kloet et al., 1990; Lattin and Romero, 2015; Romero, 2002). In males, we found slightly increased baseline corticosterone levels in testosterone-treated individuals, while in females the stress-induced

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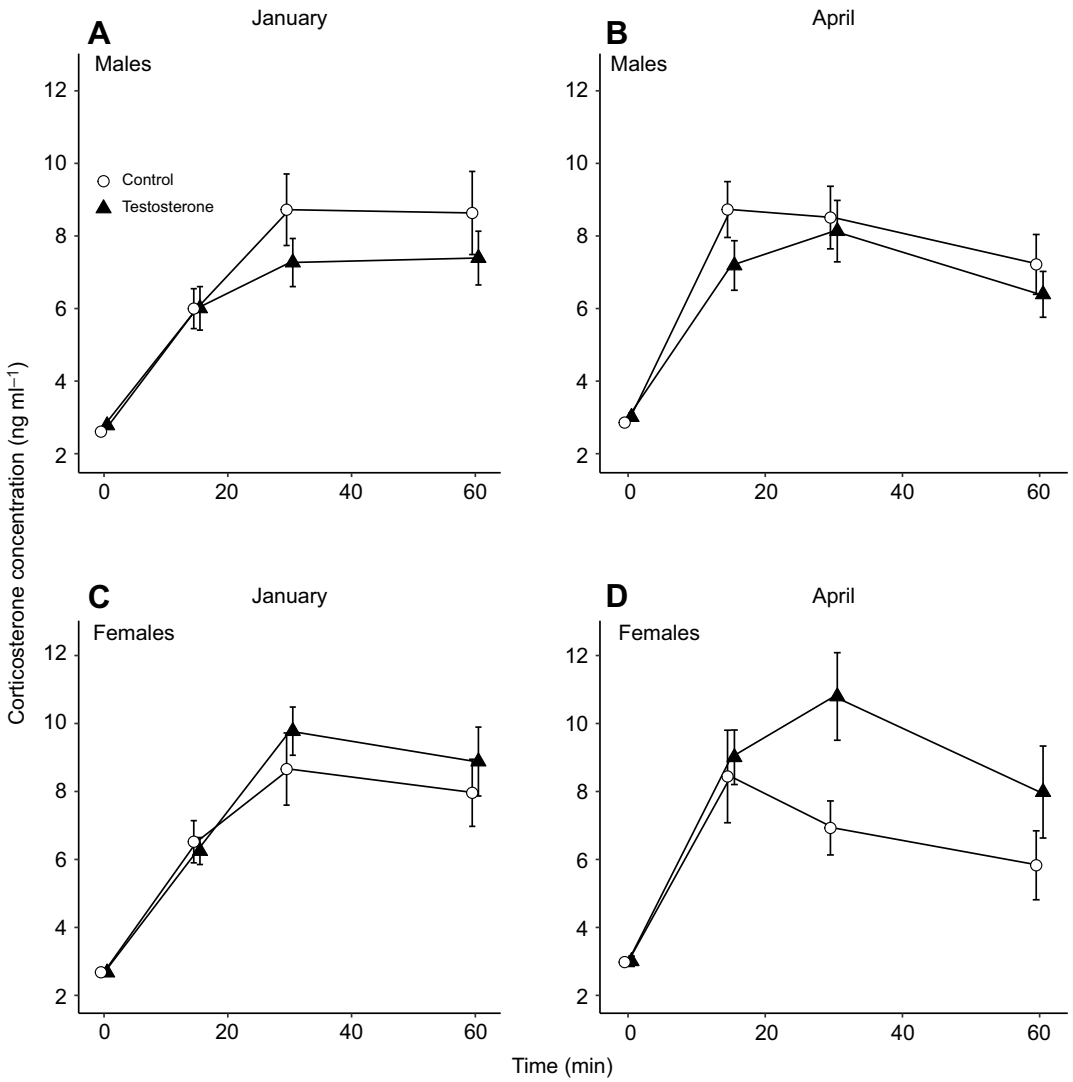

Fig. 2. Corticosterone levels in *Passer domesticus*. Plasma concentrations (mean±1 s.e.m.) of baseline (time=0 min) and stress-induced corticosterone during a 60 min standard capture and handling stress protocol of male (A,B) and female (C,D) house sparrows hatched from testosterone-treated and control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

corticosterone secretion profile, but not baseline corticosterone, was affected by *in ovo* testosterone treatment. We have no explanation for the mechanism by which developmental testosterone exposure might program increased baseline corticosterone secretion in males, but a likely explanation for the changed corticosterone stress secretion profile of females may be modification of the negative feedback system for down-regulation of stress-induced corticosterone (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 downregulation and upregulation of brain GRII 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 testosterone- 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 not, however, detect differences in ovarian follicle size (present study) or circulating levels of ovarian steroids (progesterone, testosterone, 5α-DHT and 17β-estradiol) between adult females from testosterone-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 corticosterone in males and prolonged secretion of corticosterone during stress episodes in females could affect performance (Arnold et al., 2016; Bonier et al., 2009a,b; Patterson et al., 2014; Vitousek et al., 2014). This programming might cause some of the behavioral effects of yolk testosterone that we reported previously (Partecke and Schwabl, 2008; Strasser and Schwabl, 2004); one could also speculate that the modified stress corticosterone secretion profile of females during the reproductive phase is related to a reduced mortality risk of testosterone-treated compared with 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 corticosterone levels in response to 30 min of standardized capture and handling stress (Lendvai et al., 2007) and, in females, 30 min stress-induced corticosterone levels were negatively related to inquisitive behavior in response to novelty, a component of coping style (Lendvai et al., 2011).

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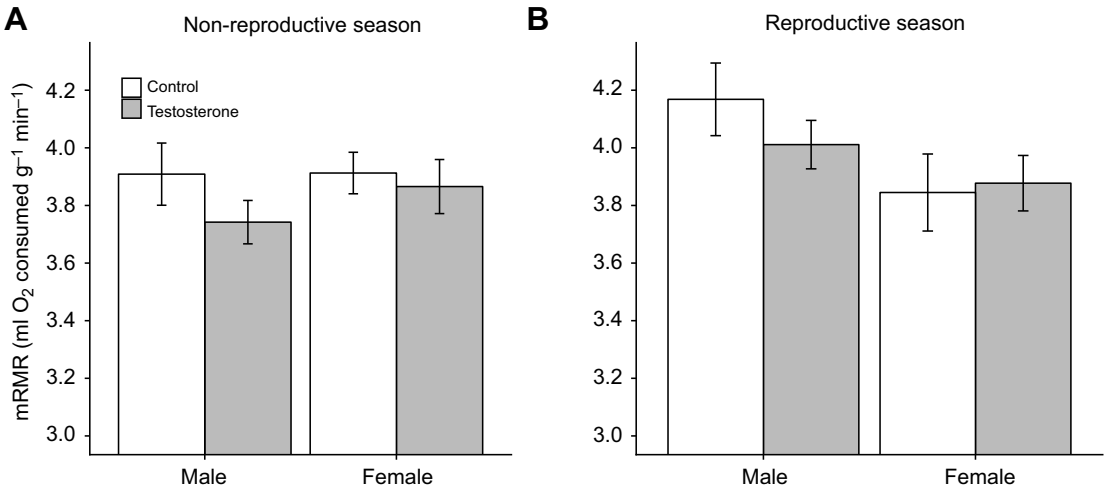

Fig. 3. Mass-specific resting metabolic rate in *Passer domesticus*. Mass-specific resting metabolic rate (mRMR; mean±1 s.e.m.) during the non-reproductive (January, A) and reproductive (April, B) phase of male and female house sparrows hatched from testosterone-treated and control eggs. January: controls: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

Our *in ovo* testosterone treatment did not affect adult mRMR. This outcome differs from experimental studies of two other passerine bird species, domesticated zebra finch (*T. guttata*) and wild pied flycatcher (*F. hypoleuca*), which both reported enhanced adult metabolic rate by *in ovo* androgen treatment [testosterone in the zebra finch (Nilsson et al., 2011); testosterone plus androstenedione in the pied flycatcher (Ruuskanen et al., 2013)]. A third study conducted with the non-passerine black-headed gull (*Larus ridibundus*) did not, however, detect any effects of egg testosterone 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 testosterone treatment on mRMR in our study indicates that the observed effects of yolk testosterone 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 testosterone-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, 2009; but see Buehler et al., 2012). We found a positive correlation of mRMR with the integrated overall corticosterone secretion during a stress episode, but not with baseline corticosterone levels. Although we did not measure corticosterone 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 that triggered a corticosterone stress secretion response similar to that measured in the stress protocol. In this case, our results might suggest that developmental testosterone 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 testosterone 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 corticosterone levels were affected in males but not females, and stress corticosterone levels were affected 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 (testosterone) 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* corticosterone (elevated as a consequence of enhanced maternal HPA activity and stress) can influence offspring HPA activity (Haussmann 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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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.S., J.P.; Methodology: H.S., J.P.; Validation: J.P.; Formal analysis: H.S., J.P.; Investigation: J.P.; Resources: H.S.; Data curation: H.S., J.P.; Writing - original draft: H.S.; Writing - review & editing: H.S., J.P.; Visualization: J.P.; Supervision: H.S.; Project administration: J.P.; Funding acquisition: H.S., J.P.

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