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ORIGINAL PAPER

Long-lasting effects of yolk androgens on phenotype in the pied flycatcher (*Ficedula hypoleuca*)

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Abstract The hormonal environment during early development, such as maternally derived androgens in bird eggs, shapes the development of the offspring in ways that may have important long-term consequences for phenotype and behavior and, ultimately, fitness. We studied the long-term effects of yolk androgens on several phenotypic and physiological traits in male and female pied flycatchers (*Ficedula hypoleuca*) by experimentally elevating yolk androgen levels and rearing birds in common-garden environment in captivity. We found that high yolk androgen levels increased the basal metabolic rates in both females and males in adulthood. High yolk androgen levels did not affect male melanin coloration or plumage ornaments, or timing or speed of moult in either sex. No effect of androgen treatment on cell-mediated or humoral immune response was found in either sex. Covariation among the measured phenotypic traits was further not altered by androgen treatment. Our results suggest that exposure to high androgen levels can have long-lasting effects on some offspring traits, but do not seem to lead to different phenotypes. Furthermore, the role of yolk androgens affecting sexually selected male traits in our study species seems to be minor. The fitness consequences of yolk androgen-induced higher metabolic rates remain to be studied.

Keywords Maternal effect · Testosterone · Bird · Moult · Immune defense · Basal metabolic rate

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Introduction

The effect of maternal phenotype on offspring phenotype is referred to as maternal effects (Mousseau and Fox 1998). In various taxa, maternal effects seem to have long-lasting consequences on adult phenotype and fitness, sometimes with adaptive consequences (reviewed in Bernardo 1996; Mousseau and Fox 1998; Galloway 2005; Groothuis et al. 2005; Gil 2008; Hasselquist and Nilsson 2009). In particular, hormonally mediated maternal effects have gained increasing interest in recent years due to their various effects on offspring traits and their potential role as a mechanism mediating life history trade-offs in many taxa (Dufty et al. 2002; Andersson et al. 2004; Groothuis et al. 2005; Uller et al. 2007). To study these effects of the early hormonal environment, egg-laying animals, such as birds, are excellent model organisms, as yolk hormone levels can be easily measured and experimentally manipulated (Groothuis and von Engelhardt 2005). Most previous experimental studies on yolk androgens have focused on their effects on early stages of life (offspring growth, development, and behavior; Uller and Olsson 2003; Groothuis et al. 2005; Uller et al. 2007; Navara and Mendonça 2008). They may however have organizational effects, for example, by modifying receptor sensitivity in different tissues towards the same hormones later in life (reviewed in Carere and Balthazart 2007; Groothuis and Schwabl 2008), which can lead to long-term effects (see below).

Recent studies have shown that yolk androgens may affect, for example, adult behavior (Strasser and Schwabl 2004; Eising et al. 2006; Tschirren et al. 2007; Ruuskanen and Laaksonen 2010) and morphological and plumage characteristics (Strasser and Schwabl 2004; Eising et al. 2006; Rubolini et al. 2006; Müller et al. 2008, 2009; Müller and Eens 2009, but see, e.g., Ruuskanen et al. 2012a). Especially, melanin-based plumage coloration seems to be androgen-dependent (Hall 1969), and high exposure to yolk androgens has been found to increase the size of melanin-based ornaments in sparrows (Strasser and Schwabl 2004). In gulls, high yolk androgen levels also speed up the development of nuptial plumage (Eising et al. 2006). Yolk androgens can also have long-term consequences on adult physiology, such as immune function (Rubolini et al. 2007; Tobler et al. 2010), metabolic rates (Nilsson et al. 2011), or female reproduction (Rubolini et al. 2007; Rutkowska et al. 2007; Müller et al. 2009), but these effects are poorly known in other than domestic birds and captive-bred populations. The changes in adult behavior, plumage traits, or physiology could affect survival and reproductive success (via, e.g., altered mating success or reproductive physiology; Partecke and Schwabl 2008; Müller and Eens 2009; Müller et al. 2009) leading potentially to fitness consequences (Ruuskanen 2010; Ruuskanen et al. 2012b).

Furthermore, if yolk androgens affect male traits (e.g., ornaments or behavior) that are sexually selected, it has been hypothesized that females could increase the attractiveness of their sons via differential yolk androgen deposition, especially when they are mated to low-quality/less attractive males (i.e., compensatory adjustment). This could be an adaptive maternal strategy in a population where more conspicuous (attractive) males have an advantage in reproduction. There is indeed evidence that yolk androgen deposition is related to male quality or attractiveness (Gil et al. 1999, 2006; Groothuis et al. 2005; Gil 2008), and in some cases, high yolk androgen levels are deposited when pairing with a potentially lower quality male (Michl et al. 2005; Navara et al. 2006; Laaksonen et al. 2011), which lends support to the hypothesis.

We studied the effects of yolk androgens on several phenotypic and physiological traits in the pied flycatcher (*Ficedula hypoleuca*) by experimentally elevating yolk androgen levels in full clutches and housing the birds in captivity in a common-garden environment. By studying multiple traits and covariation among the measured traits, we investigated whether yolk androgens may lead to different phenotypes. We investigated the long-term effects of yolk androgens on several plumage traits (melanin coloration and ornaments) and timing and speed of moult. On the basis of previous studies (Strasser and Schwabl 2004; Eising et al. 2006), we predicted that androgen-treated males would moult into darker coloration than controls and that moult would be advanced in the androgen-treated individuals. In our study species and population, as well as in a closely related species, the collared flycatcher (*Ficedula albicollis*) females deposit higher yolk androgen levels when paired with young males (Michl et al. 2005; Laaksonen et al. 2011), which could be lower quality males compared to older males that have gone through a longer selection period (Forslund and Pärt 1995). If yolk androgens enhance sexually selected male traits, this could be a female strategy to increase the attractiveness of their sons. We further measured long-term effects of yolk androgens on adult immune function (both cell-mediated and humoral immunity, challenged with novel antigens) and basal metabolic rate (BMR; oxygen consumption, both in the autumn and winter). We predicted on the basis of previous literature that birds with elevated yolk androgen level would have lower immune capacity but higher BMR than control birds (Tobler et al. 2007, 2010; Nilsson et al. 2011). We further predicted that males would have higher BMR than females, and that BMR would be higher during simulated migration in the autumn than in the wintering period (McKechnie 2008).

Methods

Study species

The pied flycatcher is a small (12–13 g), migratory, insectivorous, hole-nesting passerine bird (Lundberg and Alatalo 1992). It breeds over a large range in Europe and winters in western Africa. Pied flycatchers are single-brooded, and the modal clutch size is five to six eggs. Pied flycatcher is an abundant species that easily accepts artificial nest boxes, and thus, it is a common model species in studies of avian ecology and evolution. The dorsal coloration of male pied flycatchers varies from dull brown to black (Lundberg and Alatalo 1992). Males have also white forehead and wing patches, which are highly variable features of the breeding plumage of male pied flycatchers associated with pairing success and breeding performance (Sirkiä and Laaksonen 2009; Sirkiä et al. 2010). The UV coloration of males is another plumage trait which female pied flycatchers use in mate selection (Siitari et al. 2002; Sirkiä and Laaksonen 2009), and this trait also affects the probability of the male losing paternity in his nest (Lehtonen et al. 2009). Females are always brown and less variable than males (Lundberg and Alatalo 1992). Both sexes moult partially (contour feathers, a variable part of their wing coverts, tertials, and 0–3 innermost secondaries) on the wintering grounds in Africa around mid-February to mid-March before departure to spring migration. Summer moult (postnuptial moult, a complete moult concerning nearly all feather tracts) occurs from mid-June to mid-August, males moulting ca. 2 weeks

earlier than females (own data; Lehikoinen 2001; Salewski et al. 2004).

Field and hormone manipulation protocol

The study was conducted in 2008–2009 in a nest box breeding population in Turku, Finland (60°26.055′N, 22° 10.391′E). Full description of the field and hormone manipulation protocol is found in a study on the behavioral effects of yolk androgens on the same birds (Ruuskanen and Laaksonen 2010). On the estimated day of clutch completion, each clutch was randomly assigned to either the androgen or the control treatment. All the eggs in a single clutch were treated the same way. In the androgen treatment (initial, N=31 clutches), the eggs were injected with 7.5 ng of testosterone (Fluka) and 53.1 ng of androstenedione (Fluka) dissolved in 4 μl sesame oil. In the control treatment (initial, N=25 clutches), the eggs were injected only with 4 μl of sesame oil. The amount of injected androgens was calculated using previous data on natural yolk androgen levels from 24 clutches in the same population (testosterone mean±SD, 4.6±2.1 ng/yolk; maximum, 12.1 ng/yolk; androstenedione mean±SD, 35.6±17.0 ng/yolk; maximum, 88.6 ng/yolk; T. Laaksonen and E. Möstl, unpublished data). The amount injected corresponds to the difference between the mean and maximum values of androgens per yolk, ensuring that the final yolk androgen level in the androgen-treated clutches was on average at the upper limit of the natural range. The injected amount of androgens corresponded to 3.57 and 3.12 times the SD in testosterone and androstenedione, respectively. The position of the yolk was visualized using a light source positioned beneath the egg. The surface of the egg was first cleaned with 95 % ethanol, and a small hole was made using a disposable 27-G needle. The oil vehicle was injected into the yolk using a 25-μl Hamilton microliter syringe (702RN) and 26-G needle. After the injection, the hole in the eggshell was sealed with a drop of tissue adhesive (Vet-Seal, B. Braun Medical, Switzerland). The eggs were returned to the nest immediately after the injections. The nests were checked on the following 2 days, and the seventh and eighth eggs, if present, were likewise injected, according to the clutch treatment. This method of manipulating yolk androgen levels has been successfully used and validated in previous studies (Tschirren et al. 2005; Ruuskanen et al. 2009; Ruuskanen and Laaksonen 2010).

Average laying date of nests from the control treatment was 22.5±3.4 (SD) and of androgen treatment nests, 22.3± 3.5 (1=first of May). Average clutch size was 7.1±0.8 (SD) and 7.0±0.73 in control and androgen treatment nests, respectively. The hatching success (mean±SD) was 70.3± 18.1 % for the control treatment group and 76.1±17.1 % for the androgen treatment group. There was no significant difference in hatching success between the groups (χ^2= 1.10, p=0.29). The hatching success (mean±SD) of non-injected clutches in this population is 90.5±15.4 % (N=181, data from years 2004 to 2006; T. Laaksonen, unpublished data), thus indicating somewhat lower hatching success due to injection protocol. The nests were monitored to record hatching date as a part of population monitoring. On the fifth day after hatching, the chicks were ringed, and blood samples were taken from the brachial vein to collect DNA for molecular sexing, which was carried out during the nestling period. Full description of the molecular sexing is found in Ruuskanen and Laaksonen (2010).

Housing in the aviaries

For a full description of the housing conditions, see Ruuskanen and Laaksonen (2010). Twelve days after hatching, two chicks—one male and one female—from each of 30 broods [15 androgen-treated (AT) and 15 controls (CO)] were transferred to the aviaries. These 60 nestlings were arranged into ten new foster broods, with a brood size of six chicks. Siblings were placed in the same foster brood, with each foster brood including chicks from both treatments. Each brood was fed in the aviaries by two foster parents until it was certain that the young could forage independently (at an age of about 40 days), after which, the foster parents were released. Two aviaries were thereafter connected, and the birds were kept in groups of 12 individuals (6 males and 6 females, a roughly equal number of birds from both treatments) until late spring next year. Temperature and photoperiod were gradually changed to mimic the changes during autumn migration, wintering, spring migration, and spring in breeding grounds [temperature, 15 °C (day)/+10 °C (night) in autumn, gradually changed to constant +20 °C in winter and again to +15 °C/+10 °C in spring]. Survival of the birds in captivity was high: only three individuals were lost during the whole year.

Measurements

Plumage traits

All plumage traits were measured in spring 2009 after birds had completed the prenuptial (pre-breeding) moult. Melanin coloration of the males was measured as Drost score (score ranging from I to VII, according to Drost 1936) and percentage of black (Lundberg and Alatalo 1992). The area of the white patches in the forehead (males only) and wing (both sexes) was measured by taking digital photos with a millimeter scale at the side (at the same level; see Sirkiä and Laaksonen 2009). Photos were analyzed with a graphics program (ImageJ) using the scale in each photo. Each photo was measured twice, and average of the two measurements was used in the subsequent analyses. The repeatability for

area measurement of forehead patch (repeated measurements from one photo per individual) was 0.96 ($F_{27, 53}$=48.96, p< 0.001) and for the wing patch it was 0.99 ($F_{53, 107}$=166.64, p<0.001; Lessells and Boag 1987). Repeatability of measurements from two different photos is also high for the wing patch size (from another set of photos, r=0.98, N=82 birds, P. Järvistö and T. Laaksonen, unpublished data). UV coloration was measured from the middle tertial feather, which is in the center of the white wing patch. We measured UV coloration from this feather because white feathers typically have higher UV reflectance than dark feathers (Eaton and Lanyon 2003) and the middle tertial has a rigid structure, whereas the dark or brown body feathers on the back have a soft semi-plume structure. Furthermore, the UV of this feather is strongly correlated with that of the whole white wing patch (Sirkiä and Laaksonen 2009). The UV chroma of the white wing patch [proportion of reflectance in the UV spectrum, i.e., (reflectance at 320–400 nm)/(reflectance at 320–700 nm)] was measured in the lab with a spectrophotometer (Avantes 2048, Avantes DH-S light source; white standard WS-2).

Moult assessment

In order to assess the development of prenuptial moult, the birds were monitored in the spring for first signs of moulting. We estimated progress of moult in all birds five times during the spring [roughly 2-week intervals: 26.3, 4.4, 14.4/16.4, 5.5, and 18.5]. On 26.3, we were only able to assess 18 individuals because of time constraints, but on all other occasions, we captured and measured all birds (N=57 or 58). Body mass was also recorded. From each bird, the moult of tertials, secondaries, primaries, and greater coverts were scored according to Ginn and Melville (1983) and Jenni and Winkler (1994), where old feathers scored 0, growing feathers got a score from 1 to 4, and new feathers a score of 5. In a regular partial pre-breeding moult, all tertials, inner secondaries, 4–8 greater coverts (sometimes 0–9), and body feathers are moulted (Jenni and Winkler 1994; Salewski et al. 2004). Wings of all birds were photographed, and photos were used to later confirm the scores. We estimated the scores from both wings and used the sum of both wings, thus the maximum for tertials being 30 and for greater coverts, 100, respectively. For secondaries, only the three innermost were analyzed (max score 30 if both wings are included). We analyzed the two feather areas separately, as tertial moult was often completed (only seven individuals did not complete) within the study period, but moult of greater coverts was more variable. For reasons unknown to us, most of the birds also started postnuptial moult, i.e., moulting some of the primaries and outermost greater coverts (Jenni and Winkler 1994) late in the spring. We excluded this second moult cycle when calculating the indices for prenuptial (winter) greater covert moult. To crudely estimate the start of moult and speed of moult, we calculated linear regressions (intercept and slope) for each individual. As moult scores have often been found to increase linearly (e.g., Ojanen and Orell 1982; Ginn and Melville 1983), we think that this approach is adequate for our purpose. Linear regressions were calculated using two to four measurements from each individual (median, 3), separately for tertials and greater coverts. In the regressions, we only included observations from birds that were actively moulting. In further statistical analyses on effects of yolk androgens on moulting, we used two kinds of responses, which were calculated from linear regressions from each individual: (1) *timing of moult*, i.e., date when individual was estimated to have reached 50 % of the maximum moult score (median date) and (2) *speed of moult*, i.e., the number of days from moult start to median date (see above). Timing and speed of moult were not correlated for greater coverts (Pearson r=−0.05, p=0.69), but for tertials, timing and speed were positively correlated (Pearson r=0.39, p= 0.003). Due to the onset of postnuptial moult, we could not estimate extent of prenuptial moult in our study. All moult measurements were conducted by one experienced person (EL) to avoid variability in the method of estimation.

Basal metabolic rate

(a) General protocol

BMR is the minimum maintenance metabolism of a normothermic resting endotherm, measured at thermoneutral environmental temperatures and in the absence of thermoregulatory, digestive, circadian, or other increments in metabolic heat production (McNab 1997). Measurements were conducted twice: at the age of ca. 2 months (mean±SD, 66±1.8 days, August 2008) and at the age of ca. 7 months (mean±SD, 218± 1.7 days, January 2009), using a customized setup (see below). The first measuring period overlapped with the simulated autumn migration [+15 °C (day), +10 °C (night), ca. 15L:9D], and the second overlapped with the simulated wintering period (constant, +20 °C, 12L:12D).

(b) Respirometry setup

As proxy for metabolic rate, oxygen consumption rates of flycatchers were determined using a customized setup adapted to simultaneously measure oxygen consumption of six birds. The air delivery system to the animal chambers comprised two compressors (GAST Manufacturing Inc., Benton Harbor, MI, USA) constantly supplying room air in excess of 10 l min^{-1} and >2 bar to eight mass flow controllers (MKS Instruments, Andover, MA, USA). The flow controllers were adjusted to provide a constant air flow of

approximately 1,000 $ml\,min^{-1}$ at ambient pressure to the six animal chambers and two reference channels. Flow rate for each chamber was individually calibrated before and after each overnight oxygen consumption measurement and ranged, among chambers, from 967 to 1,099 $ml\,min^{-1}$. Differences in flow rates between calibration for individual chambers were always below 4.4 % (mean=0.28 %, SD=1 %, n=120). Animal chambers (volume, 2.03 l; inner diameter, 150 mm; height, 115 mm) were constructed from commercial drainage pipe end pieces with O-ring sealed screw lids (DYKA 160, DYKA GmbH, Wilhelmshorst, Germany). Air inlet and outlet connections were provided by short pieces of copper pipe (outer diameter, 7 mm) glued into holes drilled in the chamber walls. Chambers were closed by gluing PVC plates over the open ends. By unscrewing the lids of the pipe end caps, birds could be easily inserted or removed. Chambers were operated in two sets of three chambers (channels 1–3 and 5–7) with one reference channel each (channel 4 and 8). The outflows of the two sets of four channels were directed to two series of four three-way magnetic valves (three-way subminiature solenoid valves series 8325, ASCO, Florham Park, NJ, USA), the outflows of which in turn were connected in a way, that by sequentially switching between valves, only the air flow from one channel of each set (chamber or reference channel) was directed to a sensor, while the three remaining channels were just vented. Gas flow to the oxygen (S102) and CO_2 sensors (S154, Qubit Systems Inc., Kingston, ON, Canada) were kept independent of chamber flow rates (~1 $l\,min^{-1}$) and constant at approximately 200 $ml\,min^{-1}$ by subsampling the magnetic valve outflow using two Qubit flow systems in pull mode (P650 and P1500 Gas Pump, F900 and F1600 Flow Meter, Qubit Systems Inc., Kingston, ON, Canada). For the first set of chambers, only an oxygen sensor, and for the second set, an oxygen and a CO_2 sensor were used. An additional sensor for temperature and humidity was installed in the outflow line of chamber 6. Room temperature was also monitored by a temperature sensor (D203, Qubit Systems, Kingston, ON, Canada) placed close to the animals chamber. Gas sensors were calibrated before and after each measurement. Oxygen sensors were calibrated using a gas mixture of 90 % air/10 % N_2 (technical purity, Oy AGA Ab, Espoo, Finland) and 100 % air. For calibrating the CO_2 sensor, pure N_2, and 90 % and 100 % air, as for oxygen sensor calibrations, were applied. Due to the excellent long-term stability of the CO_2 sensor, absolute calibrations using defined gas mixtures from 0 % to 0.1 % CO_2 generated with Wösthoff precision gas mixing pumps (2 M 303/a-F, Wösthoff, Bochum, Germany) were performed only before and after each experimental run (August 2008, January 2009) of approximately 10 days. Monitoring of all electronic signals and control of magnetic valves switching cycles was performed by using a standard PC running Windows 98 with A/D acquisition card (DAS1602, Keithley Instruments Inc., Taunton, MA, USA) and TestPoint data acquisition software (TestPoint 4.1, Capital Equipment Corporation, Billerica, MA, USA). Data acquisition rate was 10 Hz with averages of signals stored to hard disk every 50 values, resulting in an effective sampling rate of 0.2 Hz.

(c) Experimental procedure and data calculation

Calibration of equipment (flow rate, gas sensors) was started in the late afternoon. Food was removed from the aviaries approximately 1 h before measurements to ensure that measurements were not confounded by digestion. At dusk, birds were caught in the aviaries (by hand or using a sweep net), weighed, and put into the measuring chambers. At each measurement round, all six birds were caught from one aviary and selected so that roughly the same number of birds from both treatments and sexes were included. The system was then set to switch between chambers every 15 min, cycling through all four channels (three chambers, one reference channel) in 1 h. The automatic measurement cycles were then stopped the next morning, birds were removed, weighed, and returned to the aviaries, and calibrations were again performed. Time spent in measurement chambers was 10 h (from 21 p.m. to 7 a.m.) in the first measuring period (autumn) and 13 h (from 19 p.m. to 8 a.m.) in the second measuring period (winter).

Oxygen consumption values were calculated from the reduced oxygen partial pressure recorded from animal chamber outflow compared to ambient oxygen partial pressure. During conversion, corrections were made for changes in ambient pressure (mean value for each daily measuring period from 1600 to 1000 hours the following morning), humidity (mean value for whole measuring periods of 10 days from data recorded with humidity sensor in chamber 6, 35 % RH in August 8, 20 % RH in January 9), and temperature (mean value of indoor temperature over whole measuring periods of 10 days, 25 °C in August 8, 25.5 °C in January 9). Values for ambient air pressures were acquired from the closest meteorological station from the Finnish Meteorological Institute covering the respective measuring periods. Since handling stress and diurnal rhythms cause increases or changes in metabolic rate, the two lowest oxygen consumption values recorded during the overnight measurement of each individual bird were averaged as representative of standard metabolic rate.

Immunological measurements

(a) Cell-mediated immunity

Cell-mediated immunity was measured in May 2009 at the age of ca. 11 months. Cellular immune function was assessed by challenging the birds with an injection of phytohemagglutinin (PHA, Sigma, code L8754) in the wing web, a frequently used method in avian ecology (Smits et al. 1999). Injected subcutaneously, PHA produces inflammation and a local swelling, caused by accumulation of T-lymphocytes, macrophages, and other leucocytes (Martin et al. 2006). The thickness of the right wing web was first measured three times with a thickness gauge (Mitutoyo 700-117SU), and the place was marked with a permanent marker. We then injected 0.2 mg of PHA dissolved in 0.04 ml of PBS in the wing web, and the swelling in the wing web was again measured after 24 h (Smits et al. 1999; Pitala et al. 2009). The size of the swelling induced by the PHA injection (post- minus pre-injection value) was used as an estimate for cell-mediated immune responsiveness. Measurements were conducted always by the same person (SR).

(b) Humoral immunity

Humoral immunity was assessed by immunization with tetanus-diphtheria vaccine following the protocol in Ilmonen et al. (2003). The measurements were made in adult birds in May 2009 at the age of ca. 11 months. This immunization was conducted at least 48 h after the PHA injection, as after this time the PHA response disappears (Smits et al. 1999). Briefly, before the immunization, birds were blood sampled from the brachial vein (70–100 μl; control sample). Birds were then given an intramuscular injection in the pectoral muscle with 100 μl of diphtheria-tetanus vaccine [Finnish National Public Health Institute; diphtheria 6.25 Lf (limit of flocculation) and tetanus 6.25 Lf, mixed with the adjuvant aluminum phosphate, 0.5 mg/ml]. Nine days later, birds were blood sampled again to measure antibody titers against tetanus toxoid. The peak response should occur around this period (see, e.g., Svensson et al. 1998, maximum titers at 10–14 days post-injection), and this sampling period has been used in previous studies with passerines (Ilmonen et al. 2003; Eeva et al. 2005; Moreno et al. 2005). Blood samples were centrifuged to separate plasma and red blood cells. Antibody concentrations in the plasma were determined using an indirect enzyme-linked immunosorbent assay (ELISA). Due to low sample volume and resource constraints, only antibodies produced against tetanus toxoid were analyzed. However, these antibodies are known to be highly correlated (see, e.g., Svensson et al. 1998). The wells were coated with 50 μl of tetanus toxoid (Calbiochem, Cat No. 582231, 0.5 Lf/ml in carbonate buffer, pH9.6) and incubated 2× overnight (o/n) at +4 °C. Hereafter, the wells were masked with 100 μl 1 % BSA-PBS (1 h at room temperature, RT) and washed three times with 200 μl of 0.05 % Tween 20 in 1× PBS. Samples were diluted in 1:150. As a standard, we used plasma of tetanus-diphtheria-vaccinated black grouse. The undiluted standard was given an arbitrary concentration of one million units of immunoglobulin per milliliter (1,000,000 U/ml). Duplicates of samples and standards (50 μl) were incubated 3 h in RT and thereafter washed again three times with 0.05 % Tween 20 in 1× PBS. After the wash, the conjugated secondary antibody (Sigma A-9171, anti-chicken IgG whole molecule, alkaline phosphatase conjugated, 1:4,000 dilution; 50 μl) was added. Thereafter, the plates were incubated (o/n) at +4 °C, washed again (3×400 μl 0.05 % Tween 20 in 1× PBS), and substrate (100 μl 1-mg/ml *p*-nitrophenylphosphate, Sigma 104-0) was added. After 60 min incubation, absorbances were measured at 450 nm (Multiskan Ascent, Therma Oy, Finland). The results are expressed as units per milliliter, and in the analyses, the difference between post- and pre-immunization has been used. For some individuals, we did not get both pre- and post-immunization blood samples or blood sample was not large enough, which reduced the sample size in the statistical analyses.

Statistical analyses

All analyses were conducted with SAS version 9.2. Wing patch size and speed of moult (for both tertials and greater coverts) were log-transformed for normality. Effect of androgen treatment on male black-brown coloration (Drost score and percentage of black) was analyzed with non-parametric Kruskal–Wallis test. All other response variables, i.e., male forehead patch size, male UV chroma, wing patch size, timing and speed of moult, cell-mediated immunity, and humoral immunity were analyzed using mixed models (proc MIXED) with treatment, sex, and their interaction (when applicable) as explanatory variables. In the analyses of cell-mediated immunity and humoral immunity, we also included body mass as a covariate. In all mixed model analyses, rearing aviary was added as a random factor to control for the rearing conditions.

In the analyses of oxygen consumption, repeated measures model was used to control for non-independence of the two measurements of the same individual. The explanatory factors in this model were treatment, sex, measuring period (autumn or winter), and all their interactions. Chamber identity (nested within measuring period) was included as a random factor to control for potential differences among the six measuring chambers. Rearing aviary was added as a random factor to control for the rearing conditions.

Normality of the residuals of all models was checked. Kenward–Roger method was used to calculate degrees of

freedom of the fixed effects (Littell et al. 2006). We used a backward model selection procedure, removing interactions, covariates, and main effects, starting with the least significant (at significance level >0.05). To confirm the non-significance of the removed interactions and main effects, each term was added to the final model separately. As our main interest in these analyses is the sex-specific differences among treatments, we present all data including the interaction factor. However, results remained the same if all non-significant factors were removed. To investigate whether covariation among the measured traits differed among treatments, we further ran general linear mixed models with each trait as the response variable at a time and another trait, along with treatment, sex (when applicable), and all their interactions as the explanatory variables. Analyses of covariation with BMRs were conducted separately for autumn and winter measurements.

Results

Averages of the measured phenotypic traits for each treatment and sex are presented in Tables 1 and 3.

Effects of high yolk androgen levels on adult plumage traits

Melanin coloration of the males (either Drost score or percentage of black) or UV chroma of the white wing patch were not affected by androgen treatment (Tables 1 and 2). The size of the white forehead patch of males was not affected by androgen treatment either (Tables 1 and 2). Size of the white wing patch was larger in males than females (mean±SD: males, 177.6±59.6 mm^2; females, 72.2±17.9 mm^2), but was not affected by the androgen treatment in either sex (Tables 1 and 2).

Compared to naturally occurring prenuptial moult in the pied flycatchers, moult of captive birds was rather similar (contour feathers, tertials, and 0–3 innermost secondaries, Jenni and Winkler 1994), but they seemed to moult a larger number of greater coverts (mean±SD, 8.5±2.1) than reported from the wild (4–8, median 6; Jenni and Winkler 1994). They also started moulting more than a month later than reported from the birds in Africa (from an unknown breeding population; Salewski et al. 2004). Timing or speed of moult of tertials or greater coverts was not affected by androgen treatment, sex, or their interaction (Tables 3 and 4).

Effects of high yolk androgen levels on adult physiological traits

Androgen-treated individuals had higher BMRs (measured as oxygen consumption per minute) than control individuals, independent of sex and measuring period (Table 2 and Fig. 1a, b). BMRs furthermore tended to be generally higher in the autumn than in the winter (marginal means of oxygen consumption per minute±SE: autumn, 42.6±1.06; winter, 39.7±1.10; Table 2 and Fig. 1a, b). There was no general sex difference in BMRs (Table 2 and Fig. 1a, b). Cell-mediated immune response (PHA) or humoral immune response (i.e., concentration of antibodies against tetanus vaccination) was not affected by androgen treatment in either sex (Tables 1 and 2).

Covariation among the measured phenotypic traits

Covariation among any of the measured phenotypic traits did not differ between androgen-treated and control groups in either sex (p values>0.05 for all treatment×covariate and sex×treatment×covariate interactions). However, there were some general correlations among the measured traits: male coloration (percentage of black) was positively correlated with UV chroma of the white wing patch and wing patch size (UV: β±SE, 739±210; $F_{1,\ 24.5}$=12.35; p=0.001; wing patch, 0.39±0.07; $F_{1,\ 25.3}$=30.42; p<0.001) and negatively with cell-mediated immune response (β±SE, −54.4±25.1; $F_{1,\ 28}$=4.60; p=0.039). UV chroma of the white wing patch was further positively correlated with wing and forehead patch size (wing patch: β±SE, 0.0002±0.00007; $F_{1,\ 23.7}$=11.87; p=0.002; forehead patch: β±SE, 0.001±0.0004; $F_{1,\ 22.5}$=9.66; p=0.005) and negatively with cell-mediated immune response (β±SE, −0.049±0.021; $F_{1,\ 23.8}$=5.48; p=0.028). BMR (either in autumn or winter) or humoral immune

Table 1 Plumage traits and physiological traits (mean±SD) in relation to androgen treatment and sex

Sex	Treat	Drost	% Black	UV chroma	Forehead PA (mm^2)	Wing PA (mm^2)	CMI (mm)	Humoral imm (U/ml[a])
Male	AT	5.47±1.60	25.3±31.3	0.191±0.02	13.4±8.2	168.6±61.1	0.40±0.21	10.47±17.04
Male	CO	5.36±1.27	24.6±23.0	0.187±0.02	15.1±9.8	187.3±58.7	0.38±0.17	7.88±14.91
Female	AT					72.5±14.7	0.34±0.18	−1.46±10.15
Female	CO					72.0±20.6	0.35±0.22	8.13±10.77

N=10–15 per group depending on the measurement. Drost scale: 1–7 (7=brown, 1=black). Cell-mediated and humoral immunity are expressed as the difference between post- and pre-immunization

PA patch area, *CMI* cell-mediated immunity

[a] Humoral immunity is expressed as concentration of antibodies, 10^4 U/ml

Table 2 Effect of androgen treatment on plumage traits and physiological measurements

	ndf, *ddf*	F/χ^2	*p*
Drost (males only), *N*=28			
Treatment		0.15	0.70
% Black (males only), *N*=28			
Treatment		0.33	0.57
Forehead patch (males only), *N*=28			
Treatment	1, 24.9	0.3	0.59
UV chroma (males only), *N*=28			
Treatment	1, 23.5	0.23	0.56
Wing patch size, *N*=54			
Treatment	1, 48	0.28	0.60
Sex	1, 48.1	132.41	<0.0001
Treatment×sex	1, 46.1	0.6	0.44
Oxygen consumption, *N*=115			
Treatment	1, 91	6.85	0.01
Sex	1, 106	0.64	0.42
Treatment×sex	1, 106	0.06	0.80
Measuring period	1, 102	3.58	0.06
Cell-mediated immunity, *N*=54			
Treatment	1, 49.9	0.01	0.91
Sex	1, 51	0.71	0.40
Treatment×sex	1, 47.8	0.11	0.74
Humoral immunity, *N*=48			
Treatment	1, 41.2	0.93	0.34
Sex	1, 41.5	1.76	0.19
Treatment×sex	1, 40.5	2.49	0.12

For all χ^2 tests *df*=1. In the model of oxygen consumption, a repeated measures analysis was used as birds were measured at two measuring periods

response was not associated with any of the measured phenotypic traits (all *p* values>0.05). There were no correlations among other measured traits ($p>0.05$).

Discussion

We studied the long-term effects of early androgen exposure on multiple adult phenotypic and physiological traits in pied flycatchers in a common-garden experiment. We found that high yolk androgen levels increased adult BMRs in both males and females. However, high yolk androgen levels were not associated with melanin coloration, plumage ornaments, or timing or speed of prenuptial moult. They neither affected adult cell-mediated or humoral immune response. Yolk androgens thus have long-lasting effects on adult physiology, with potential fitness consequences, but they do not seem to create distinctly different phenotypes. As yolk androgens did not affect sexually selected male traits, they do not seem to act as means for female pied flycatchers to enhance the attractiveness of their sons.

Our study is one of the few showing that high yolk androgen levels lead to higher metabolic rates in adulthood. Similar results have been found in captive zebra finches (*Taeniopygia guttata*), both in adults and in nestlings (Tobler et al. 2007; Nilsson et al. 2011; but see Eising et al. 2003), but the potential mechanisms are unclear. Exposure to high yolk androgen levels may modify hypothalamus–pituitary–gonadal axis, leading to higher circulating androgen levels (Müller et al. 2007), which have been shown to increase basic metabolic rate (Buchanan et al. 2001; Buttemer et al. 2008). Alternatively, at least in nestlings, high metabolic rates are thought to be associated with higher activity of chicks with high yolk androgen levels (Tobler et al. 2007). We also found that males from androgen treatment were also more active than controls as adults (Ruuskanen and Laaksonen 2010), which may explain the higher metabolic rates of adults to some extent. Parallel to earlier studies, we further found that metabolic rates varied among seasons, being higher in autumn during migration period than in the winter (reviewed in, e.g., McKechnie 2008). Differences among autumn and winter should not be due to different duration of the measuring period, as lowest values occurred in early morning in both periods (in August, on average, at 3:40 o'clock and in January at 3:00 o'clock).

The fitness consequences of yolk androgen-induced higher metabolic rates are yet unknown. Depending on the environmental conditions, higher energetic needs caused by high metabolic rates could potentially lead even to a survival cost. Even small increases in metabolic rate could play an important role in harsh conditions when food is limiting. Furthermore, higher metabolic rates may be associated with

Table 3 Timing of moult (median date of moult progress, from linear regressions, 1=1st of March) and estimate of moult speed (number of days from moult start date to median date of moult progress) in relation to androgen treatment and sex, separately for tertials and greater coverts

N=12–15 per group

		Timing of moult		Speed of moult	
Sex	Treatment	Tertials Mean±SD	Greater cov Mean±SD	Tertials Mean±SD	Greater cov Mean±SD
Male	AT	46.0±14.2	55.4±10.8	20.3±5.2	28.2±10.8
Male	CO	50.3±14.0	55.8±11.3	17.3±2.4	26.7±15.1
Female	AT	51.3±16.8	59.0±13.3	20.2±7.3	29.7±7.0
Female	CO	53.9±16.1	60.0±18.3	19.0±7.8	26.2±10.2

Table 4 Effects of androgen treatment on timing of moult (median date of moult progress) and speed of moult (number of days from estimated moult start date to median date of moult progress) separately for tertials and greater coverts

	ndf, *ddf*	*F*	*p*
Timing: tertials, *N*=55			
Treatment	1, 50.3	0.69	0.41
Sex	1, 47.1	1.15	0.28
Treatment×sex	1, 47.1	0.04	0.85
Timing: greater coverts, *N*=54			
Treatment	1, 49.2	0.06	0.81
Sex	1, 46	1.10	0.30
Treatment×sex	1,46.1	0.01	0.92
Speed: tertials, *N*=55			
Treatment	1, 48.6	1.44	0.23
Sex	1, 47.1	0.00	0.98
Treatment×sex	1, 47.1	0.22	0.63
Speed: greater coverts, *N*=54			
Treatment	1, 47.9	1.57	0.21
Sex	1, 46.2	0.22	0.63
Treatment×sex	1, 46.2	0.44	0.51

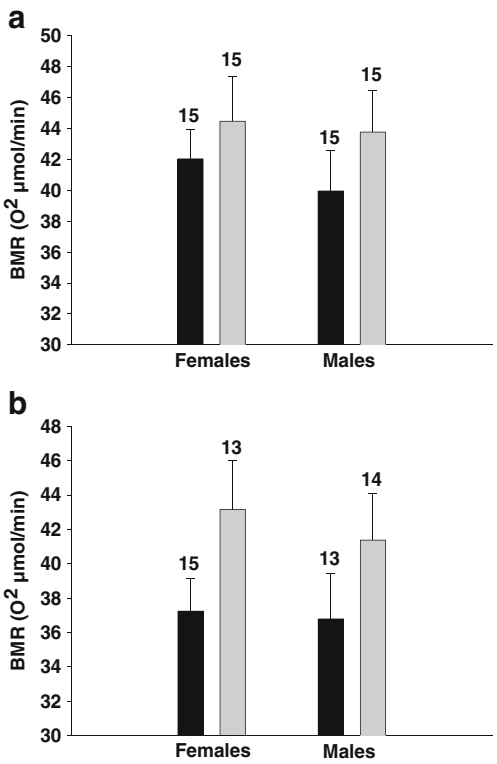

Fig. 1 Basal metabolic rate (mean+SD; micromoles oxygen consumption per minute) in yolk androgen-treated (*grey bars*) and control (*black bars*) males and females: (**a**) autumn and (**b**) winter. Sample sizes are shown *above the bars*

overproduction of free oxygen radicals, leading to oxidative damage of lipids, proteins, and DNA (Perez-Campo et al. 1998; but see Barja 2007; reviewed in, e.g., Costantini 2008; Metcalfe and Alonso-Alvarez 2010). Interestingly, in a previous study with collared flycatchers (*F. albicollis*), a closely related species to pied flycatchers, we found low local recruitment rate of androgen-treated male offspring, but the causes of low recruitment were unclear (Ruuskanen 2010; Ruuskanen et al. 2012b). If high yolk androgen levels indeed caused higher metabolic rates also in this species, it may explain our previous results to some extent. To get an idea of the biological relevance of the BMR differences found among treatment groups in this study (on average 11 %, but in some comparisons up to 15 %), we can compare these differences to differences found among other breeding stages. For example, egg laying has been found to increase metabolic rate 16–27 % and chick-feeding 20 % (see, e.g., Nilsson and Råberg 2001; Vezina and Wingfield 2005), whereas mounting an immune response elevated metabolic rate by ca. 5 % (Nilsson et al. 2007). This indicates that the differences found among treatment groups in our study can have biological significance.

Another potential fitness cost of high yolk androgen levels may be associated with their immunosuppressive effects: high yolk androgen levels have been found to suppress both humoral and cell-mediated immune system in nestling birds, although the overall picture is not very clear, as many studies have not found any significant effects (Groothuis et al. 2005; Gil 2008). Furthermore, high circulating androgen levels may be immunosuppressive, but also here the effects vary (reviewed in, e.g., Roberts et al. 2004). However, only few studies have investigated the long-term effects of high yolk androgen levels on immunity. Parallel to our results, Cucco et al. (2008), Tobler et al. (2010), and Bonisoli-Alquati et al. (2011b) did not find any long-term effects of androgens on cell-mediated immune response in adult grey partridges (*Perdix perdix*), zebra finches, or pheasants (*Phasianus colchicus*), respectively. However, in contrast to our results, humoral immune response was higher in androgen-treated zebra finches than controls (Tobler et al. 2010). The role of yolk androgens modifying adult immune response and its potential fitness consequences should thus be further investigated. Furthermore, the lack of differences among treatment groups in physiological parameters in our study must be carefully interpreted: The fact that moulting pattern was altered from natural pattern may suggest that also timing of reproductive physiology in the spring may have been different than under natural conditions. However, we assume that in our common-garden environment, any effects of artificial environment should be similar in both treatment groups, and thus differences among groups would be apparent also in these conditions.

In contrast to earlier studies and our predictions (Strasser and Schwabl 2004; Eising et al. 2006), we found no effect of

androgen treatment on melanin-based coloration. Yolk androgens did not affect other secondary sexual male traits either (forehead or wing patch sizes or UV chroma of the white wing patch), and the covariation among plumage and physiological traits was not altered by androgen treatment. We also found a general negative correlation between melanin coloration and immune response that is in opposite direction to the previously published results (Jacquin et al. 2011). Furthermore, opposite to the results of yolk androgens in gulls (Eising et al. 2006) and previous studies manipulating circulating hormonal levels (Ketterson et al. 1992; Duttmann et al. 1999; Clotfelter et al. 2004), timing or speed of moult was not affected by androgen treatment. Thus, our results imply that in the pied flycatcher, yolk androgens are unlikely to affect sexual selection via male plumage characteristics. A recent meta-analysis concluded that the effects of yolk androgens on male phenotypic traits and sexual selection are low (Müller and Eens 2009), suggesting that yolk androgens are likely to cause only minor variation in androgen- and non-androgen-dependent sexually selected traits. This may be explained by the many other environmental and/or genetic factors influencing the expression of sexually selected male characters (such as condition dependence, developmental stress, trade-offs with reproductive effort, and parasitism, e.g., Hamilton and Zuk 1982; Andersson 1986). To our knowledge, two studies have directly tested the effect of egg androgen treatment on male attractiveness using mate choice tests: control females copulated more with yolk androgen-treated males than control males in pheasants (Bonisoli-Alquati et al. 2011a), but not in zebra finches (Rutkowska et al. 2007). Furthermore, in captive canaries (*Serinus canaria*), females mated with males from androgen-treated eggs increased their investment in clutch size and clutch mass: this may however indicate either that females assess androgen-treated males as more attractive or that they compensated for lower mate quality or future offspring viability (Müller et al. 2008). Taken together, our results and previous findings suggest that it is unlikely that differential allocation of yolk androgens into eggs could be a female strategy to increase the attractiveness of sons via effects on secondary sexual traits. At least it is unlikely in our study species. However, our own results and other studies have showed that some aspects of male behavior are affected by yolk androgens (Ruuskanen and Laaksonen 2010), and thus, the importance of yolk androgens modifying behavior in the context of mate choice should be further studied.

Conclusions

We found that exposure to high androgen levels in the egg lead to increased BMRs in both males and females in adulthood. Yolk androgens thus have long-lasting, potentially organizing effects on adult physiology. One of the puzzling questions in yolk androgen research is the extensive variation in yolk androgen levels among and within clutches, one explanation being that a cost of high yolk androgen levels, either for the mother or the offspring, may explain the high variation. Higher metabolic rates induced by high yolk androgen levels and their potential energetic/fitness costs could be one factor explaining the varying allocation in eggs.

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Ethical standards The rearing of the birds in captivity and all experiments were conducted under license from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESLH-2008-03693) and the Environmental Center of Southwestern Finland (license number LOS-2007-L-264-254). All experiments comply with the current laws of Finland.

References

Andersson M (1986) Evolution of condition-dependent sex ornaments and mating preferences—sexual selection based on viability differences. Evolution 40:804–816

Andersson S, Uller T, Lohmus M, Sundström F (2004) Effects of egg yolk testosterone on growth and immunity in a precocial bird. J Evol Biol 17:501–505

Barja G (2007) Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. Rejuv Res 10:215–223

Bernardo J (1996) Maternal effects in animal ecology. Am Zool 36:83–105

Bonisoli-Alquati A, Matteo A, Ambrosini R, Rubolini D, Romano M, Caprioli M, Dessi-Fulgheri F, Baratti M, Saino N (2011a) Effects of egg testosterone on female mate choice and male sexual behavior in the pheasant. Horm Behav 59:75–82

Bonisoli-Alquati A, Rubolini D, Caprioli M, Ambrosini R, Romano M, Saino N (2011b) Egg testosterone affects wattle color and trait covariation in the ring-necked pheasant. Behav Ecol Sociobiol 65:1779–1790

Buchanan KL, Evans MR, Goldsmith AR, Bryant DM, Rowe LV (2001) Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? Proc R Soc Lond B 268:1337–1344

Buttemer WA, Warne S, Bech C, Astheimer LB (2008) Testosterone effects on avian basal metabolic rate and aerobic performance: facts and artefacts. Comp Biochem Physiol A 150:204–210

Carere C, Balthazart J (2007) Sexual versus individual differentiation: the controversial role of avian maternal hormones. Trends Endocr Met 18:73–80

Clotfelter ED, O'Neal DM, Gaudioso JM, Casto JM, Parker-Renga IM, Snajdr EA, Duffy DL, Nolan V, Ketterson ED (2004) Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? Horm Behav 46:171–178

Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett 11:1238–1251

Cucco M, Guasco B, Malacarne G, Ottonelli R, Tanvez A (2008) Yolk testosterone levels and dietary carotenoids influence growth and immunity of grey partridge chicks. Gen Comp Endocr 156:418–425

Drost R (1936) Über das Brutkleid männlicher Trauerfliegenfänger, *Muscicapa hypoleuca* (Pall.). Vogelzug 6:179–186

Dufty AM, Clobert J, Møller AP (2002) Hormones, developmental plasticity and adaptation. Trends Ecol Evol 17:190–196

Duttmann H, Dieleman S, Groothuis TGG (1999) Timing of moult in male and female shelducks *Tadorna tadorna*: effects of androgens and mates. Ardea 87:33–39

Eaton MD, Lanyon SM (2003) The ubiquity of avian ultraviolet plumage reflectance. Proc R Soc Lond B 270:1721–1726

Eeva T, Hasselquist D, Tummeleht L, Nikinmaa M, Ilmonen P (2005) Pollution related effects on immune function and stress in a free-living population of pied flycatcher *Ficedula hypoleuca*. J Avian Biol 36:405–412

Eising CM, Visser GH, Müller W, Groothuis TGG (2003) Steroids for free? No metabolic costs of elevated maternal androgen levels in the black-headed gull. J Exp Biol 206:3211–3218

Eising CM, Müller W, Groothuis TGG (2006) Avian mothers create different phenotypes by hormone deposition in their eggs. Biol Lett 2:20–22

Forslund P, Pärt T (1995) Age and reproduction in birds—hypotheses and tests. Trends Ecol Evol 10:374–378

Galloway LF (2005) Maternal effects provide phenotypic adaptation to local environmental conditions. New Phytol 166:93–99

Gil D (2008) Hormones in avian eggs: physiology, ecology and behavior. Adv Stud Behav 38:337–398

Gil D, Graves J, Hazon N, Wells A (1999) Male attractiveness and differential testosterone investment in zebra finch eggs. Science 286:126–128

Gil D, Ninni P, Lacroix A, De Lope F, Tirard C, Marzal A, Moller AP (2006) Yolk androgens in the barn swallow (*Hirundo rustica*): a test of some adaptive hypotheses. J Evol Biol 19:123–131

Ginn HB, Melville DS (1983) Moult in birds. British Trust for Ornithology, Tring

Groothuis TGG, Schwabl H (2008) Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? Philos T Roy Soc B 363:1647–1661

Groothuis TGG, von Engelhardt N (2005) Investigating maternal hormones in avian eggs: measurement, manipulation, and interpretation. Ann NY Acad Sci 1046:168–180

Groothuis TGG, Müller W, von Engelhardt N, Carere C, Eising C (2005) Maternal hormones as a tool to adjust offspring phenotype in avian species. Neurosci Biobehav R 29:329–352

Hall PF (1969) Hormonal control of melanin synthesis in birds. Gen Comp Endocr 2:451–458

Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds—a role for parasites. Science 218:384–387

Hasselquist D, Nilsson JA (2009) Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. Philos T Roy Soc B 364:51–60

Ilmonen P, Hasselquist D, Langefors A, Wiehn J (2003) Stress, immunocompetence and leukocyte profiles of pied flycatchers in relation to brood size manipulation. Oecologia 136:148–154

Jacquin L, Lenouvel P, Haussy C, Ducatez S, Gasparini J (2011) Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. J Avian Biol 42:11–15

Jenni L, Winkler R (1994) Moult and ageing of European passerines. Academic, London

Ketterson ED, Nolan V, Wolf L, Ziegenfus C (1992) Testosterone and avian life histories—effects of experimentally elevated testosterone on behavior and correlates of fitness in the dark-eyed junco (*Junco hyemalis*). Am Nat 140:980–999

Laaksonen T, Adamczyk F, Ahola M, Möstl E, Lessells CM (2011) Yolk hormones and sexual conflict over parental investment in the pied flycatcher. Behav Ecol Sociobiol 65:257–264

Lehikoinen E (2001) Varpuslintujen sulkasadon tutkimus. Moult of Finnish passerines—an overview. Linnut-vuosikirja 2000. BirdLife Suomi, Helsinki, pp 55–65

Lehtonen PK, Primmer CR, Laaksonen T (2009) Different traits affect gain of extrapair paternity and loss of paternity in the pied flycatcher, *Ficedula hypoleuca*. Anim Behav 77:1103–1110

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities—a common mistake. Auk 104:116–121

Littell RC, Milliken GA, Stroup WW, Wolfinger RD (2006) SAS system for mixed models. SAS Institute Inc., Cary

Lundberg A, Alatalo R (1992) The pied flycatcher. Poyser, London

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299

McKechnie AE (2008) Phenotypic flexibility in basal metabolic rate and the changing view of avian physiological diversity: a review. J Comp Physiol B 178:235–247

McNab BK (1997) On the utility of uniformity in the definition of basal rate of metabolism. Physiol Zool 70:718–720

Metcalfe NB, Alonso-Alvarez C (2010) Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct Ecol 24:984–996

Michl G, Török J, Péczely P, Garamszegi LZ, Schwabl H (2005) Female collared flycatchers adjust yolk testosterone to male age, but not to attractiveness. Behav Ecol 16:383–388

Moreno J, Morales J, Lobato E, Merino S, Tomas G, Martinez-de la Puente J (2005) Evidence for the signaling function of egg color in the pied flycatcher *Ficedula hypoleuca*. Behav Ecol 16:931–937

Mousseau TA, Fox CW (1998) Maternal effects as adaptations. Oxford University Press, New York

Müller W, Eens M (2009) Elevated yolk androgen levels and the expression of multiple sexually selected male characters. Horm Behav 55:175–181

Müller W, Deptuch K, Lopez-Rull I, Gil D (2007) Elevated yolk androgen levels benefit offspring development in a between-clutch context. Behav Ecol 18:929–936

Müller W, Vergauwen J, Eens M (2008) Yolk testosterone, postnatal growth and song in male canaries. Horm Behav 54:125–133

Müller W, Vergauwen J, Eens M (2009) Long-lasting consequences of elevated yolk testosterone levels on female reproduction. Behav Ecol Sociobiol 63:809–816

Navara KJ, Mendonça MT (2008) Yolk androgens as pleiotropic mediators of physiological processes: a mechanistic review. Comp Biochem Physiol A 150:378–386

Navara KJ, Hill GE, Mendonça MT (2006) Yolk androgen deposition as a compensatory strategy. Behav Ecol Sociobiol 60:392–398

Nilsson JÅ, Råberg L (2001) The resting metabolic cost of egg laying and nestling feeding in great tits. Oecologia 128:187–192

Nilsson JÅ, Granbom M, Råberg L (2007) Does the strength of an immune response reflect its energetic cost? J Avian Biol 38:488–494

Nilsson JF, Tobler M, Nilsson JÅ, Sandell MI (2011) Long-lasting consequences of elevated yolk testosterone for metabolism in the zebra finch. Physiol Biochem Zool 84:287–291

Ojanen M, Orell M (1982) Onset of moult among breeding pied flycatchers (*Ficedula hypoleuca*) in Northern Finland. Vogelwarte 31:445–451

Partecke J, Schwabl H (2008) Organizational effects of maternal testosterone on reproductive behavior of adult house sparrows. Devel Neurobiol 68:1538–1548

Perez-Campo R, Lopez-Torres M, Cadenas S, Rojas C, Barja G (1998) The rate of free radical production as a determinant of the rate of

aging: evidence from the comparative approach. J Comp Physiol B 168:149–158

Pitala N, Ruuskanen S, Laaksonen T, Doligez B, Tschirren B, Gustafsson L (2009) The effects of experimentally manipulated yolk androgens on growth and immune function of male and female nestling collared flycatchers *Ficedula albicollis*. J Avian Biol 40:225–230

Roberts ML, Buchanan KL, Evans MR (2004) Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim Behav 68:227–239

Rubolini D, Romano M, Martinelli R, Leoni B, Saino N (2006) Effects of prenatal yolk androgens on armaments and ornaments of the ring-necked pheasant. Behav Ecol Sociobiol 59:549–560

Rubolini D, Martinelli R, von Engelhardt N, Romano M, Groothuis TGG, Fasola M, Saino N (2007) Consequences of prenatal androgen exposure for the reproductive performance of female pheasants (*Phasianus colchicus*). Proc R Soc Lond B 274:137–142

Rutkowska J, Wilk T, Cichoń M (2007) Androgen-dependent maternal effects on offspring fitness in zebra finches. Behav Ecol Sociobiol 61:1211–1217

Ruuskanen S (2010) Maternal effects in birds—the ecological and evolutionary significance of yolk androgens and other egg components. Painosalama Oy, Turku

Ruuskanen S, Laaksonen T (2010) Yolk hormones have sex-specific long-term effects on behavior in the pied flycatcher (*Ficedula hypoleuca*). Horm Behav 57:119–127

Ruuskanen S, Doligez B, Tschirren B, Pitala N, Gustafsson L, Groothuis TGG, Laaksonen T (2009) Yolk androgens do not appear to mediate sexual conflict over parental investment in the collared flycatcher *Ficedula albicollis*. Horm Behav 55:514–519

Ruuskanen S, Doligez B, Gustafsson L, Laaksonen T (2012a) Long-term effects of yolk androgens on phenotype and parental feeding behavior in a wild passerine. Behav Ecol Sociobiol 66:1201–1211

Ruuskanen S, Doligez B, Pitala N, Gustafsson L, Laaksonen T (2012b) Long-term fitness consequences of high yolk androgen levels: sons pay the costs. Funct Ecol 26:884–894

Salewski V, Altwegg R, Erni B, Falk KH, Bairlein F, Leisler B (2004) Moult of three Palaearctic migrants in their West African winter quarters. J Ornithol 145:109–116

Siitari H, Honkavaara J, Huhta E, Viitala J (2002) Ultraviolet reflection and female mate choice in the pied flycatcher, *Ficedula hypoleuca*. Anim Behav 63:97–102

Sirkiä PM, Laaksonen T (2009) Distinguishing between male and territory quality: females choose multiple traits in the pied flycatcher. Anim Behav 78:1051–1060

Sirkiä PM, Virolainen M, Laaksonen T (2010) Melanin coloration has temperature-dependent effects on breeding performance that may maintain phenotypic variation in a passerine bird. J Evol Biol 23:2385–2396

Smits JE, Bortolotti GR, Tella JL (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572

Strasser R, Schwabl H (2004) Yolk testosterone organizes behavior and male plumage coloration in house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 56:491–497

Svensson E, Råberg L, Koch C, Hasselquist D (1998) Energetic stress, immunosuppression and the costs of an antibody response. Funct Ecol 12:912–919

Tobler M, Nilsson JA, Nilsson JF (2007) Costly steroids: egg testosterone modulates nestling metabolic rate in the zebra finch. Biol Lett 3:408–410

Tobler M, Hasselquist D, Smith HG, Sandell MI (2010) Short- and long-term consequences of prenatal testosterone for immune function: an experimental study in the zebra finch. Behav Ecol Sociobiol 64:717–727

Tschirren B, Saladin V, Fitze PS, Schwabl H, Richner H (2005) Maternal yolk testosterone does not modulate parasite susceptibility or immune function in great tit nestlings. J Anim Ecol 74:675–682

Tschirren B, Fitze PS, Richner H (2007) Maternal modulation of natal dispersal in a passerine bird: an adaptive strategy to cope with parasitism? Am Nat 169:87–93

Uller T, Olsson M (2003) Prenatal exposure to testosterone increases ectoparasite susceptibility in the common lizard (*Lacerta vivipara*). Proc R Soc Lond B 270:1867–1870

Uller T, Astheimer L, Olsson M (2007) Consequences of maternal yolk testosterone for offspring development and survival: experimental test in a lizard. Funct Ecol 21:544–551

Vezina F, Wingfield TD (2005) The metabolic cost of egg production is repeatable. J Exp Biol 208:2533–2538