UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS
Departamento de Zoología y Antropología Física

TESIS DOCTORAL

Yolk androgens as modulators of life history trade-offs in the spotless starling ("Sturnus unicolor")

Los andrógenos de la yema como moduladores de los compromisos de las estrategias vitales en el estornino negro ("Sturnus unicolor")

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Jaime A. Muriel

Directores
Diego Gil Pérez
Lorenzo Pérez Rodríguez

Madrid, 2016

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Tesis Doctoral

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Memoria presentada para optar al grado de Doctor por

Jaime Alejandro Muriel Redondo

Bajo la dirección de los Doctores

Diego Gil Pérez y Lorenzo Pérez Rodríguez

Madrid – Enero 2016

Diego Gil Pérez, Científico Titular del Museo Nacional de Ciencias Naturales – CSIC

Lorenzo Pérez Rodríguez, Investigador Postdoctoral Contratado "Severo Ochoa" de la Estación Biológica de Doñana – CSIC

CERTIFICAN:

Que la presente memoria titulada "Los andrógenos de la yema como moduladores de los compromisos de las estrategias vitales en el estornino negro *Sturnus unicolor*" que para optar al grado de Doctor presenta **Jaime Alejandro Muriel Redondo**, ha sido realizada bajo nuestra dirección en el Departamento de Ecología Evolutiva del Museo Nacional de Ciencias Naturales – CSIC (Madrid). Esta memoria está además adscrita académicamente al Departamento de Zoología y Antropología Física de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid. Considerando que representa trabajo suficiente para constituir una Tesis Doctoral, autorizamos su presentación.

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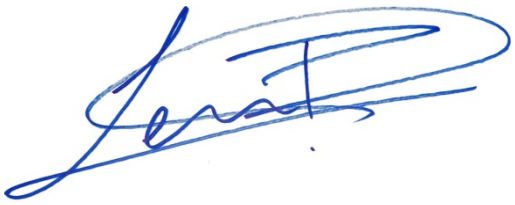

Diego Gil Pérez Lorenzo Pérez Rodríguez

El Doctorando: Jaime Alejandro Muriel Redondo

En Madrid, a 15 de enero de 2016

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“Natura semina scientiæ nobis dedit, scientiam non dedit.”

“Nature has given us the seeds of knowledge, not knowledge itself.”

“La naturaleza nos ha dado las semillas del conocimiento, no el conocimiento mismo.”

Seneca—Epistolæ Ad Lucilium. CXX.

Lucius Annaeus Seneca

(Corduba, 4 a. C. – Roma, 65 d. C.)

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Durante estos seis años que he pasado en el Museo he tratado de aprovechar al máximo esta gran oportunidad de realizar la tesis en uno de los centros de investigación biológica más destacados de España (MNCN-CSIC). Sin embargo, a uno siempre le asaltan las dudas de que si se podrían haber jugado mejor las cartas, o de si haciendo unas cosas y no otras se hubiera optimizado el tiempo. Pero una cosa que no dudo ni por un segundo es de haber invertido mi tiempo en conocer a gente tan maravillosa como los que han sido mis amigos "los becarios"… En primer lugar quisiera agradecer a **Carlos P.** el haberme guiado hacía el apasionante mundo de la ornitología, ya que allá por el 2004/2005 me animaste a ir a la campaña de anillamiento de Salburua con Alejandro O. y Azaitz U., donde realmente empezó mi verdadera vocación por estos seres emplumados. Gracias además por todas esas sesiones de anillamiento que hemos compartido en la Sierra, la Autónoma o en las Minas (junto con Juancho C., Álvaro D., Óscar M… y el resto de miembros de Montícola), así como anillando rapaces y chotacabras por el Suroeste de Madrid. Fueron unos años de formación personal y profesional que nunca olvidaré, ¡¡gracias!! Agradecer también a **Alex C.** y **Juan N.** por haber tirado de mí en numerosas ocasiones para unirme al nuevo grupo de becarios que se estaba formando. Aquellos primeros pacharanes en industriales me hicieron conocer a gente realmente increíble que de otra forma hubiera sido difícil coincidir. Aunque todos y cada uno de los miembros de nuestro gran grupo de "pestuzos" (¡¡rozamos los 50!!) ocupa un lugar destacado en esta fase de mi vida, un conjunto de factores hace inevitable pasar más tiempo con

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Durante mi última temporada de campo (primavera de 2014) tuve el privilegio de vivir en El Ventorrillo (estación biológica que dispone el MNCN-CSIC en la Sierra de Guadarrama). Fue realmente gratificante poder alojarme allí, ya que pude desplazarme a mi zona de estudio cómodamente, al tiempo que me permitió organizarme y trabajar en un ambiente inmejorable. De ahí que agradezca de un modo especial a **José M.**, **Pilar L.** y **María Jesús**, el haberme facilitado todo lo necesario durante mi estancia. No sólo yo me he beneficiado de estas instalaciones, sino que

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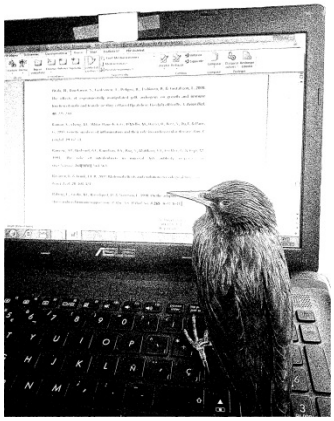


PD. No podía acabar este apartado sin dar las gracias a *Quasi*, quien además de mostrarme el valor de cada dato en una nube de puntos, cada día me enseña que la especie de estudio es mucho más que un modelo en la que basar experimentos.

ABSTRACT

Maternal effects can potentially affect ecological and evolutionary processes, improving the adaptation of offspring to variable environments. In birds, mothers can buffer offspring from the impacts of environmental heterogeneity by adjustment of resources, such as hormones, that are transmitted to the developing offspring via the egg. Exposure to steroids during early development has been shown to affect a variety of traits, leading to important fitness consequences.

Although several experimental studies have shown how nestlings benefit from increased yolk androgens through increased growth and survival, high levels of yolk androgens also involve costs. Accelerated growth induced by yolk androgens may suppress immune responses because of the trade-off between body mass gain and immune function. Manipulation of the androgen environment of an embryo may also induce a wide range of life-history trade-offs. Most previous studies have focused on the effects of yolk androgens on early life stages. However, these maternal hormones can have long-term consequences on adult phenotype and fitness.

In order to draw more accurate conclusions about the effect of yolk androgens as modulators of life-history trade-offs, one should consider that their consequences may vary depending on additional factors, such as embryo sex, developmental phase, type of androgen, dose and environmental context. Data for this thesis were obtained during the 2009-2014 breeding seasons in a nest-box population located in central Spain (Soto del Real, Madrid). We manipulated androgen levels in spotless starling eggs (*Sturnus unicolor*) and analyzed the balance of benefits and costs in early development, as well as their long term effects on adult phenotype and reproductive output.

We found that the two main androgens found in avian yolks, testosterone (T) and androstenedione (A4), exert different effects on different traits of the developing nestling. However, their effects are not additive. Yolk androgens show complex dose-response effects during early development, including both linear and non-linear responses for different nestling traits, supporting the idea that the balance of cost and benefits is strongly determined by hormone levels (i.e. higher androgens levels are not always beneficial). In addition, these cost of androgen levels, often emerge as sex-specific immunosuppression, as high hormone levels negative impact some components of the innate and adaptive axes of the immune system (e.g. lysozyme activity of the plasma and cell-mediated immunoresponsiveness) in male nestlings but not in females. Also, the effect of yolk androgens on both pre- and post-hatching development and immune function is context dependent: increased yolk androgens accelerated embryonic development and improved lymphocyte proliferation (a measure of adaptive immunity) in early clutches, but reduced nestling survival and lymphocyte proliferation in clutches laid at the end of the breeding season, when environmental conditions are harsher. Finally, yolk androgen levels not only modulated nestling development, but also exerted long term effects on key life-history traits in a sex-specific way. Hence, high yolk androgens enhanced the development of a plumage sexual ornament in this species (the

length of throat feathers), but also impaired cellular immune function, survival rate and breeding success of birds hatched in late clutches. In females, in contrast, yolk androgens did not exert long-term consequences on survival, but reduced clutch size and increased egg volume, which resulted in transgenerational effects, increasing offspring size at the F1.

Summarizing, our findings support the importance of hormone-mediated maternal effects on individual phenotypic plasticity. Variations in yolk androgen levels exert both short- and long-term effects on different life-history traits that ultimately impact on offspring fitness. Thus, this Thesis confirms the implication of maternally derived androgens on offspring performance, adding support to their role in mediating evolutionary changes.

RESUMEN

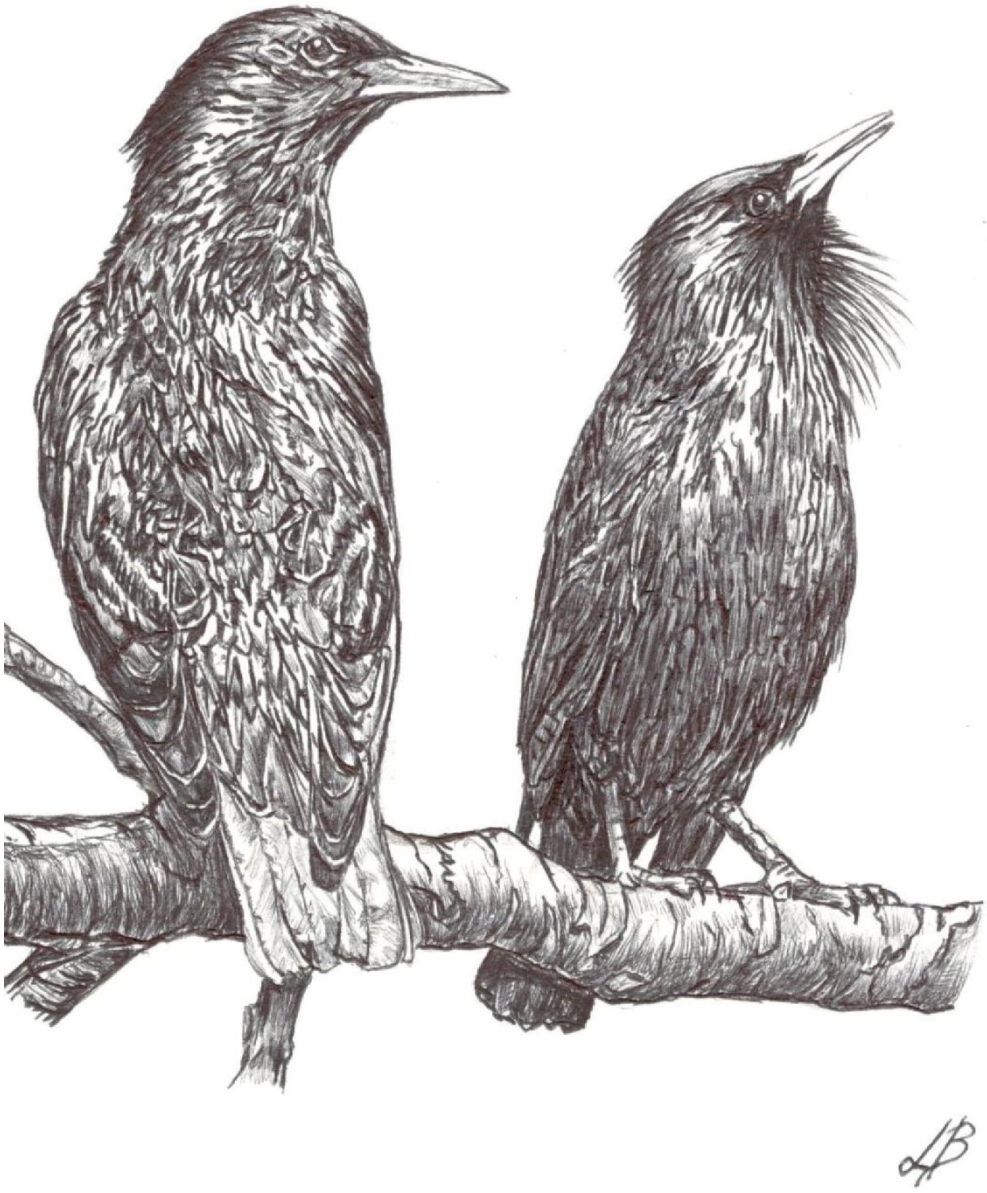
Los efectos maternos pueden intervenir en diversos procesos ecológicos y evolutivos, mejorando la adaptación de la descendencia a un ambiente cambiante. En aves, las madres pueden amortiguar el impacto de la heterogeneidad ambiental en la progenie mediante las hormonas que depositan en la yema del huevo. Aunque diversos estudios han mostrado que los andrógenos de la yema pueden beneficiar ciertos aspectos del desarrollo temprano de los pollos (incremento de la tasa de crecimiento y supervivencia, por ejemplo), unos niveles altos de estas hormonas también pueden conllevar costes para el individuo, como una inmunosupresión derivada, por ejemplo, del compromiso entre crecimiento y respuesta inmune. Aunque la mayor parte de los estudios realizados hasta ahora se han centrado en los efectos de estos andrógenos de origen materno sobre el desarrollo temprano del individuo, dichos efectos podrían tener también consecuencias a largo plazo, alterando el fenotipo y la eficacia biológica del individuo a lo largo de su vida.

Para evaluar de forma correcta el papel de los andrógenos de la yema como moduladores de los compromisos de las estrategias vitales, debemos considerar también que sus efectos pueden variar en función de factores como el sexo del embrión, la fase del desarrollo, el tipo de andrógeno en cuestión, su dosis o el contexto ambiental en que se desenvuelve el individuo. En esta Tesis Doctoral, empleando como modelo de estudio al estornino negro (*Sturnus unicolor*), manipulamos la concentración de andrógenos en yema en una población natural de la especie con el objeto de analizar el balance entre costes y beneficios durante el desarrollo temprano, así como los efectos sobre la estrategia vital de los individuos a lo largo de su vida.

Encontramos que los dos andrógenos más abundantes en la yema de huevo en aves, testosterona (T) y androstenediona (A4), afectaron a distintos rasgos del pollo durante el desarrollo en el nido y que sus efectos no son aditivos. Estos andrógenos ejercen efectos dependientes de la dosis bastante complejos durante el desarrollo temprano, que pueden ser tanto lineales como no lineales en función del rasgo de que se trate. Esto sugiere que unos niveles altos de andrógenos en la yema no siempre resultan beneficiosos para el individuo, sino que el balance entre costes y beneficios de dichas hormonas depende enormemente de su concentración. Por otro lado, un incremento en los niveles de andrógenos en la yema tuvo un efecto inmunosupresor dependiente del sexo del pollo, afectando negativamente componentes tanto del eje innato (la actividad de la lisozima plasmática) y como adaptativo (respuesta inmunitaria celular) del sistema inmunitario en machos, pero no en hembras. Además, el efecto de los andrógenos de la yema en el desarrollo previo y posterior a la eclosión fue dependiente del contexto ambiental: mayores niveles de andrógenos aceleraron el desarrollo embrionario y mejoraron la proliferación linfocitaria (un componente de la respuesta inmune adaptativa) en puestas tempranas, pero redujeron la supervivencia en el nido y la proliferación linfocitaria en puestas realizadas al final de la estación, cuando las condiciones ambientales son más duras. Finalmente, los niveles de andrógenos en la yema no sólo afectaron al desarrollo

y estado del pollo en el nido, sino que también tuvieron influencia a largo plazo sobre ciertos rasgos de su estrategia vital, de nuevo dependiendo del sexo del individuo. Así, un incremento en los niveles de andrógenos del huevo se tradujo en un mayor desarrollo de las plumas de la garganta (un ornamento sexual en esta especie), pero afectó negativamente la función inmunitaria celular, la tasa de supervivencia y el éxito reproductor en machos de puestas tardías. En hembras, en cambio, un incremento en la concentración de andrógenos en la yema no afectó a la supervivencia adulta, pero disminuyó el número de huevos puestos e incrementó su tamaño, lo que contribuyó a un efecto transgeneracional de los andrógenos maternos, aumentando el tamaño de los descendientes en la F1.

En resumen, nuestros resultados apoyan papel relevante de los efectos maternos mediados por hormonas en la plasticidad fenotípica de los individuos. Las variaciones en los niveles de andrógenos en la yema se traducen en efectos a corto y largo plazo en rasgos de la estrategia vital de los individuos que, en última instancia, tienen un impacto detectable en su eficacia biológica. Por tanto, esta Tesis Doctoral confirma la implicación de los andrógenos de origen materno en el desempeño de la descendencia, apoyando su papel mediador en los procesos de cambio evolutivo.



The courtship

The spotless starling shows a complex breeding strategy, with high rates of polygyny. Males present a group of ornamental throat feathers with enlarged and narrowed apical parts, which are much less developed in females. This sexually dimorphic plumage trait is displayed while singing and courting, as males sing with the bill raised and the feathers of the throat and breast conspicuously erected.

GENERAL INTRODUCTION

Evolution and phenotypic plasticity

In his mythic voyage to the Galapagos Islands in 1835, Charles Darwin collected 14 species of Finches that were mainly differentiated by beak size, whose shapes were adaptations to different diets available among the islands. This led him to postulate that nature had selected the most suitable beak shape against less useful ones. However, Darwin just considered natural selection as the only factor in evolutionary change, and did not propose other processes that provide variation between the members of a population. The theory was completed when the results of Gregor Mendel were brought to public light, showing that there was a recombination of parental traits in offspring. The concept of the gene was first conceived by Gregor Mendel in the 1860s, but it wasn't until 1909 that Wilhelm Johannsen coined the term "gene" to refer to discrete determiners of inherited characteristics. Thus, this Danish biologist introduced the concepts genotype and phenotype to differentiate what he thought were distinct entities. According to his view, the genotype of an organism leads to the organism's phenotype through the process of development, under the influence of the environment (Johannsen, 1911). By merging these traditions, we could understand **evolution** as a heritable *change in the properties of populations of organisms or groups of such populations, over the course of generations* (Futuyma, 1998), where the environment acts to select the most fit phenotypes.

The causes and consequences of variation in phenotype could play a leading role in the understanding of the evolutionary change of life history traits. In this sense, evolutionary biology highlights a key mechanism: **phenotypic plasticity**, which is defined as the capacity of a single genotype to exhibit a range of phenotypes in response to variation in the environment (Fordyce, 2006). Although initially phenotypic plasticity was just applied to morphological changes induced by the environment (Woltereck, 1909; Schlichting and Pigliucci, 1998), any biochemical, physiological, behavioural and life history trait could also show phenotypic plasticity (reviewed in Whitman and Agrawal, 2009). Changes in these traits might be vital to survive and reproduce above maintenance levels and, hence, are necessary for the persistence of the population in heterogeneous and variable conditions (Robinson and Dukas, 1999; Pigliucci *et al.*, 2006; Zunzunegui *et al.*, 2011). In this context, Price and coauthors specified that moderate levels of (adaptive) plasticity would optimal to perform in novel environments (Price *et al.*, 2003). This plasticity is adaptive, because individuals showing a plastic response have higher fitness than those that do not (Ghalambor *et al.*, 2007). Plasticity encompasses a diversity of environmentally induced responses that can lead to different potential evolutionary outcomes, depending on the range of phenotypes are encountered by selection (Ghalambor *et al.*, 2007; West-Eberhard, 2003). Most traits are plastic, but the degree to which plasticity is adaptive or non-adaptive depends on whether environmentally induced phenotypes are closer or further away from the local optimum (Ghalambor *et al.*, 2015). Adaptive plasticity is a ten-

dency for a genotype to express a phenotype that enhances its ability to survive and reproduce in each environment, while non-adaptive plasticity includes any response to environmental induction that does not enhance fitness (reviewed in Fitzpatrick, 2012). It may have evolved as a consequence of variable conditions experienced in the ancestral environment (Levins, 1968; Via and Lande, 1985; Sultan and Spencer, 2002; Price *et al.*, 2003). Plasticity can be active or passive, which means that it is either predominantly anticipatory or a consequence of the environment, and can be considered at the level of genes, individuals, and populations (Forsman, 2014). Because no organism is infinitely plastic there must be limits and costs that define the extension of this response (reviewed in Auld *et al.*, 2010). Phenotypic plasticity might evolve when there is a sufficient genetic variation (Via and Lande, 1987; Via *et al.*, 1995) due to genetic correlations with other traits that are under selection or to genetic drift (van Kleunen and Fischer, 2005). Theoretical models for the evolution of adaptive phenotypic plasticity predict that, given genetic variation, selection will favour adaptive plasticity when: (i) populations are exposed to variable environments, (ii) environments produce reliable cues, (iii) selection favours different phenotypes in each environment, and (iv) no single phenotype exhibits superior fitness across all environments (reviewed in Ghalambor *et al.*, 2007). Since plasticity was incorporated into the evolutionary framework, environment has been recognised not only as playing a basic role in evolution by selecting among genetically fixed phenotypic variation, but also as a force generating that variation (Gilbert and Epel, 2009; Pfennig *et al.*, 2010; West-Eberhard, 2003). In summary, phenotypic plasticity could be considered as a key mechanism in buffering natural selection, producing new trait combination and shaping ecological speciation (Thibert-Plante and Hendry, 2011).

Our understanding of phenotypic development is occasionally limited by the artificial separation of gene expression and the ontogenetic trajectory in response to environmental changes. However, this frontier can be crossed when we understand that phenotypic plasticity might be controlled by **epigenetic mechanisms** (Herman *et al.*, 2014; Varriale, 2014). Heritable variation in phenotypes, and thus potential for evolutionary change, can in principle not only be caused by variation in DNA sequence, but also by underlying epigenetic variation. The term epigenetics refers to processes that induce heritable changes in gene expression without altering the gene sequence (Bird, 2007; Frésard *et al.*, 2013). These changes are not only heritable from cell to cell through lineage development, but also during the formation of gametes through meiotic cell division, and thus may be transmitted across generations and influence the phenotype of offspring (Gilbert and Epel, 2009; Jablonka and Raz, 2009; Daxinger and Whitelaw, 2012; Varriale, 2014). Epigenetic processes are integral to determining when and where specific genes are expressed (Goldberg *et al.*, 2007); hence, alterations in these processes may lead to profound changes in phenotype. Epigenetic effects could provide a rapid source of phenotypic variation under novel conditions, which could affect the ability of populations to persist and therefore possibly affecting the performance and reproductive success of individual organisms.

Maternal effects as a singular case of phenotypic plasticity

In order to increase fitness, individuals may avail themselves of the information provided by the environment to adopt optimal strategies. In the context of natural selection, being able to take predictive information and modifying strategies would be positively selected. Recent studies indicate that in some cases environmentally induced epigenetic changes may

be inherited by future generations (Richards, 2006; Bossdorf *et al.*, 2008), which could affect the evolutionary trajectories of populations and the life history traits of organisms (Daxinger and Whitelaw, 2012; Varriale, 2014). It is in this sense that **parental effects** (usually from maternal origin) could appear as a source of epigenetic changes allowing a rapid adjustment to changing conditions. These epigenetic modifications can result in phenotypic changes at the cellular and subsequently organismal level. Changes in the parental condition that in turn affect the phenotype of their offspring create a transgenerational epigenetic (non-genetic) phenomenon that can persist over many generations (see above). As it has been reviewed by previous publications (Mousseau *et al.*, 2009; Badyaev and Uller, 2009), parental effects mean different things to different scientists and in different areas of biology. Thus, parental effects could represent discrete static components of phenotypic variance in the context of quantitative genetics (e.g., Wolf and Wade, 2009). At the same time, several studies have interpreted parental effects as adaptations (e.g., Crean and Marshall, 2009; Harris and Uller, 2009), as well as the side-effects of parent–offspring trade-offs in behavioural and evolutionary ecology (e.g., Duckworth, 2009; Russell and Lummaa, 2009). Finally, these epigenetic changes could also be interpreted as a transgenerational transfer of essential developmental templates and resources in animal physiology and developmental biology (e.g., Brown and Shine, 2009) or as an initial stage in the evolutionary process of change (Badyaev, 2009).

Within a wider evolutionary framework, parental effects allow an increase in the offspring fitness by reliably transferring developmental resources needed to reconstruct, maintain and modify genetically inherited components of the phenotype (Badyaev and Uller, 2009). Adaptive transgenerational plasticity is predicted to evolve in cases when the parental environment reliably predicts offspring environment (Agrawal *et al.*, 1999; Galloway, 2005; Marshall and Uller, 2007). Both paternal and maternal environments may contribute to such transgenerational plasticity, although maternal effects are typically greater in magnitude than paternal effects (e.g. Schmid and Dolt, 1994; Lacey, 1996; Etterson and Galloway, 2002). More specifically, **maternal effects** are considered epigenetic modifications of offspring phenotype caused by the environment provided by the mother during development (Mousseau and Fox, 1998). Adaptive maternal effects arise when the environment or phenotype of the mother influence traits linked to offspring fitness, including morphology, physiology, behaviour, and reproductive success (Bernardo, 1996a, b; Mousseau and Fox, 1998; Marshall and Uller, 2007, Wolf and Wade, 2009; Gilbert and Epel, 2009). Thus, without any change in offspring DNA sequence, mothers may adjust by phenotypic plasticity the development of their offspring to environmental conditions to increase Darwinian fitness (Mousseau and Fox, 1998). When maternal effects increase the fitness of offspring in the presence of environmental change, they are sometimes called ‘adaptive transgenerational phenotypic plasticity’ or ‘anticipatory maternal effects’ (Fox *et al.*, 1997; Agrawal *et al.*, 1999; Marshall and Uller, 2007). For example, mothers exposed to predators can produce predation-resistant offspring such that both maternal and offspring fitness are maximised (Agrawal *et al.*, 1999). Alternatively, mothers may use maternal effects to manipulate offspring for their own survival in cases of conflict. However, regardless of whether maternal effects increase or decrease survival or reproductive success of individual offspring, selection ultimately would play to maximise maternal fitness (Bernado, 1996a; Marshall and Uller, 2007). Thus maternal effects would be the result of parent-offspring interactions, whose selection

pressures sometimes have conflicting interests (Trivers, 1974).

The adaptive value of maternal effects has been demonstrated in various animal and plant taxa (reviewed by Bernardo, 1996a; Mousseau and Fox, 1998; Galloway, 2005; Groothuis *et al.*, 2005a; Hasselquist and Nilsson, 2009). Mothers could buffer offspring from the impacts of environmental heterogeneity by the adjustment of resources that may be transmitted to the developing offspring via the placenta, egg, or seed (Roach and Wulff, 1987; Schwabl, 1996a; Mousseau and Fox, 1998; Grindstaff *et al.*, 2003; Maestripieri and Mateo, 2009). In mammals, maternal factors can be transferred via the placenta, the colostrum (the milk produced for the first few days after birth which is a rich natural source of nutrients, antibodies, and growth factors for the suckling neonate) or in regular milk during lactation (Glezen, 2003; Siegrist, 2003; Lemke *et al.*, 2004). In birds, reptiles and fishes, most maternal factors are mainly transferred via the egg to the offspring (Bly *et al.*, 1986; Schumacher *et al.*, 1999; Grindstaff *et al.*, 2003), so this maternal transmission in oviparous species is mainly focussed before birth. In short, maternal effects have the potential to profoundly affect ecological and evolutionary processes, improving the adaptation of offspring to variable, but predictable, environments (Bernardo, 1996a, b; Mousseau and Fox, 1998; Fox and Savalli, 2000; Badyaev *et al.*, 2002; Rotem *et al.*, 2003; Galloway, 2005; Räsänen and Kruuk, 2007; Marshall and Uller, 2007; Galloway and Etterson, 2007).

Maternal effects in avian species: the egg value

In birds, as in other oviparous species, development of the embryo occurs within a self-contained egg environment, the characteristics of which are a direct result of the maternal condition at the time of egg formation (Price, 1998; Poisbleau *et al.*, 2009). Avian maternal effects could be related to egg size and quality (Rubolini *et al.*, 2011), as well as postnatal factors such as efficiency of incubation, nestling feeding and level of predator defence (Krist, 2011). One of the most studied proxies of female investment in eggs is probably egg size (Bernardo, 1996a; Christians, 2002), which may have direct effects on growth and survival of offspring (Williams, 1994; Amundsen, 1995; Dzialowski and Sotherland, 2004; Reed *et al.*, 2009). In addition, mothers may adjust the development and the phenotype of their offspring to environmental conditions by an adaptive allocation of resources into the egg. These maternally-derived components could be nutrients (e.g. proteins and lipids) which are important for the development of the embryo, antioxidants (e.g. yolk carotenoids) that reduce lipid peroxidation in the embryo and enhance immune function (Surai and Speake, 1998; Blount *et al.*, 2000; Royle *et al.*, 2001; Biard *et al.*, 2005), immunoglobulins which provide the primary form of humoral immune defence for the offspring (reviewed by Grindstaff *et al.*, 2003; Morales *et al.*, 2006; Boulinier and Staszewski, 2008; Hasselquist and Nilsson, 2009), albumen lysozymes as a component of the antibacterial immunity (e.g. Trziszka, 1994; Saino *et al.*, 2002b), and hormones, such as **androgens** (testosterone, androstenedione and dihydrotestosterone), estrogens and corticosterone (reviewed in Gil, 2003; Groothuis *et al.*, 2005a; Gil, 2008). These resources are often physiologically costly, and can influence nestling fitness and survival (Williams, 1994; Nager *et al.*, 2000). The concentration of these components may vary depending on environmental information perceived by the mother, such as temperature, food availability or mate quality (Kaplan, 1992; Sheldon, 2000; Benton *et al.*, 2005). Consequently the effects of egg size and its composition on offspring fitness are among the most commonly researched maternal effects, where class

Aves has been possibly the most studied taxon (Krist and Remeŝ, 2004; Krist, 2011).

Steroid hormones as modulators of maternal effects in birds

Based on an extensive literature, it can be argued that avian eggs provide excellent models to study hormone-mediated maternal effects since embryos develop outside the mother's body in relatively large eggs, facilitating descriptive and experimental studies (reviewed in Groothuis *et al.*, 2005a; Groothuis and Schwabl, 2008; Gil, 2008; Ruuskanen, 2015). Maternal hormones are important mediators of prenatal maternal effects since important and variable amounts of maternal steroids can be transferred from the mother to the offspring during egg production (Schwabl, 1993; Love *et al.*, 2005). In fact, hormone-mediated maternal effects, such as exposure to androgens during early development can exert both short- and long-term effects on various offspring traits, with potential fitness consequences (Schwabl, 1993; Lipar and Ketterson, 2000; Eising *et al.*, 2001; Andersson *et al.*, 2004; Sockman and Schwabl, 2000; Ruuskanen and Laaksonen, 2010; Ruuskanen *et al.*, 2012). Groothuis and Schwabl (2008) suggested that maternal steroids are suitable components for exploring the adaptive value of maternal effects for two main reasons: Firstly, they are integral regulatory signals in the cascade of programmed processes of development and differentiation that leads from genotype to phenotype; and secondly, they are integral mediators of phenotypic responses by adults to environmental cues and change (Groothuis and Schwabl, 2008).

In avian species, several steroid hormones have been identified in yolks, including glucocorticoids (Kozlowski and Ricklefs, 2010), estrogens (Carere and Balthazart, 2007), and androgens (Schwabl, 1993). For example, the effects of prenatal exposure to glucocorticoids, such as corticosterone, are variable between species, but may directly affect offspring fitness (Hayward *et al.*, 2004; Love *et al.*, 2005; Groothuis and Schwabl, 2008). However, most studies over the last 20 years have focused on egg androgens, mainly testosterone (T), androstenedione (A4), and 5α-dihydrotestosterone (DHT), which are single-step metabolites of the ovarian androgen synthesis pathway (Groothuis *et al.*, 2005a; Groothuis and Schwabl, 2008; Gil, 2008). Thus, A4 is converted to T by 17b-hydroxysteroid dehydrogenase (17β-HSD), but also these androgens are potentially converted to estrogens (estrone and estradiol), particularly by aromatase (CYP19). Additionally, 5α-reductase converts testosterone to 5α-DHT, an androgen which can act only via the androgen receptor and which cannot be converted to T or estrogens (Groothuis and Schwabl, 2008; Carere and Balthazart, 2007; Sanderson, 2006).

External factors such as day length, temperature, food, behaviour, or social status provide the trigger for oogenesis and ovulation (reviewed in Gil, 2008), while maternal transfer of androgens to the egg may be determined by both within- and between-clutch variation patterns (reviewed in Groothuis *et al.*, 2005a; Gil, 2008) (see below). Maternal hormones can get into the egg either by a direct incorporation from the steroidegenic cells that surround the follicle (concentric layers of tissue that surround the oocyte) while the yolk is being formed (Huang *et al.*, 1979; Johnson, 1999) or through diffusion from blood vessels or the female's internal organs before the shell is formed (reviewed in Gil, 2008). Thus we would expect to find a positive correlation between different steroids in the plasma levels of the ovulating female (estradiol, corticosterone, androgens) and yolk hormonal levels (e.g., Schwabl, 1996b; López-Rull and Gil, 2009). In this line Schwabl (1997a) reported a positive correlation between maternal faecal testosterone (as-

sumed to reflect circulating plasma levels) and yolk testosterone. However, the proportion of these steroids in the plasma levels are not always correlated with egg deposition levels, showing that there is not a simple passive mechanism from plasma to egg (Williams *et al.*, 2004). There is extensive literature on the possible mechanisms and implications of the androgen deposition to eggs (Groothuis and Schwabl, 2008; Williams, 2012). Some recent evidence suggests that there is scope for independent regulation, but it must be kept in mind that even if there is no regulation, it does not mean that hormone transfer could not be adaptive (reviewed in Ruuskanen, 2015). Although adaptive functional and evolutionary studies continue to proliferate rapidly, the underlying physiological mechanism that enables the female to regulate the steroid hormone content of her eggs has not yet been firmly established, and the source of yolk steroids itself is a question that should be rigorously addressed in future studies.

Differential allocation of yolk androgens to eggs within a clutch may allow mothers to shape the development and the competitive asymmetries within their brood (Schwabl *et al.*, 1997b). In this way, increased androgens in the last egg of asynchronously hatching clutches could compensate for age- and size-related disadvantages of the chick hatching from that egg (Lipar and Ketterson, 2000). In line with this idea, yolk androgen concentrations are often found to increase with the position of an egg in the laying sequence (Schwabl, 1993; Lipar *et al.*, 1999; Sockman and Schwabl, 2000; Eising *et al.*, 2001; Pilz *et al.*, 2003). In order to maximize offspring fitness, maternal yolk androgen concentrations not only vary across the laying sequence within clutches (Schwabl, 1993; Eising *et al.*, 2001; Schwabl *et al.*, 1997b; López-Rull and Gil, 2009) but also exhibit an even larger variation between clutches (Groothuis *et al.*, 2005a; Gil, 2008). Three main sources of variation have been found to explain differences between clutches in yolk androgen levels: female quality (e.g. Tschirren *et al.*, 2004, Tanvez *et al.*, 2007, Pilz *et al.*, 2003 but see Tobler *et al.*, 2007a; Verboven *et al.*, 2003); maternal environment , such as food availability (Verboven *et al.*, 2003; Gasparini *et al.*, 2007; Benowitz-Fredericks *et al.*, 2013), photoperiod (Schwabl, 1996b) or ectoparasite abundance (Tschirren *et al.*, 2004; Postma *et al.*, 2014); social factors affecting the mother, like aggressive interactions (Whittingham and Schwabl, 2002), mate attractiveness (Gil *et al.*, 1999, 2004; Uller *et al.*, 2005; Gwinner *et al.*, 2013), or breeding density (Schwabl, 1997a; Groothuis and Schwabl, 2002; Pilz and Smith, 2004). These factors may influence the amount of androgens deposited by females in the eggs, but also conform an external context that may affect the final balance of the physiological trade-offs mediated by yolk androgens (e.g. Martínez-Padilla *et al.*, 2014). Thus experiments testing the context- and dose-dependent effects of androgens on offspring performance are required to understand how mothers adjust the yolk androgen levels to each breeding context in order to maximize offspring fitness, from an adaptive perspective.

Maternal androgens as a mediator of life-history trade-offs

Beyond parent-offspring and sexual conflict over parental investment (Trivers, 1974; Godfray, 1995), female birds may deposit variable amounts of physiologically active substances into egg yolks (Williams, 1994; Bernardo, 1996b) in order to adjust the offspring phenotype to specific requirements (Mousseau and Fox, 1998; Giordano *et al.*, 2014). Although several experimental studies, in which fresh eggs were injected with androgens, have shown how nestlings benefit from increased yolk androgens, e.g. in terms of increased growth and survival (reviewed in Gil, 2008), high levels of yolk androgens also

involve costs (e.g. Navara *et al.*, 2005; Tobler *et al.*, 2007b). Manipulation of the androgen environment of an embryo may also induce a wide range of life-history trade-offs both in the **short- and long-term** (reviewed in Groothuis *et al.*, 2005b; Gil, 2008; Ruuskanen, 2015).

Short-term effects

Small increases in yolk androgen levels may stimulate the development of the hatching muscle during embryonic development (Lipar and Ketterson, 2000), accelerate embryonic development (Eising *et al.*, 2001; Eising and Groothuis, 2003), enhance post-natal growth rate (Schwabl, 1996a; Eising *et al.*, 2001; Pilz *et al.*, 2004; Navara *et al.*, 2005), improve competitive behaviour in nestlings (Müller *et al.*, 2009, 2012) or intensify begging behaviour (Schwabl, 1996a; Eising and Groothuis, 2003), suggesting that maternal yolk androgens are generally beneficial to offspring.

However, one might expect that prenatal exposure to androgens can also entail some negative side effects (reviewed in Navara and Mendonça, 2008), given the premises of the immunocompetence handicap hypothesis (Folstad and Karter, 1992). According to this hypothesis, androgens could be beneficial for some traits such as the production of male secondary sexual traits, but also harmful due to their immunosuppressive effects (reviewed in Owen-Ashley *et al.*, 2004; but see Roberts *et al.*, 2004; Groothuis and Schwabl, 2008). In a similar way, the accelerated growth induced by yolk androgens, may lead to suppression of immune responsiveness because of the trade-off between body mass gain and immune function. Such trade-off may arise because both processes are energetically costly and compete for the limited resources (reviewed by Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Demas, 2004), although this may not be the sole cause (reviewed in Groothuis *et al.*, 2005a; Gil, 2008). Thus, yolk androgens may mediate this trade-off, resulting in potentially negative effects on the immune system (Müller, *et al.*, 2005; see also Tschirren *et al.*, 2005). It has indeed been found that prenatal androgen overexposure may decrease cellular and humoral immune responsiveness elicited by standard in vivo challenges by lipopolysaccharides, phytohemagglutinin or sheep red blood cells in developing nestlings (Groothuis *et al.*, 2005b; Müller *et al.*, 2005a; Navara *et al.*, 2005; Sandell *et al.*, 2009). The differences reported in the literature on the relationship between experimentally manipulated levels of yolk androgens and subsequent immune responsiveness of androgen-treated nestlings could be because trade-offs between different components of the immune system itself (Martin *et al.*, 2006; Forsman *et al.*, 2008; Tobler *et al.*, 2010; Clairardin *et al.*, 2011). However, few studies have explored simultaneously the effects of yolk androgens on more than one component of the immune system (e.g. Müller *et al.*, 2005a; Sandell *et al.*, 2009).

Long-term effects

Several studies have shown that the transfer of maternal hormones to the eggs can have long-lasting consequences on adult phenotype and fitness (reviewed by Bernardo, 1996a, b; Mousseau and Fox, 1998; Galloway, 2005; Groothuis *et al.*, 2005a; Gil, 2008), supporting a potential mediating role of yolk androgens life-history trade-offs (Groothuis *et al.*, 2005b; Uller *et al.*, 2007; Gil, 2008). Although not much attention has been paid to the mechanisms underlying yolk hormone-mediated maternal effects (Groothuis and Schwabl, 2008), potential pathways may include organizational and long-lasting activational effects (Pfannkuche *et al.*, 2011), changes in the steroid production after hatching or alterations in the androgen receptor density (Pfannkuche *et al.*, 2011; Vergauwen *et al.*, 2014; but see Rubolini *et al.*, 2014).

Beyond the specific mechanisms, there is increasing evidence that yolk hormone-mediated effects generate phenotypic changes that also persist into adulthood (sexual characteristics: Strasser and Schwabl, 2004; Eising *et al.*, 2006; Rubolini *et al.*, 2006; but see Ruuskanen *et al.*, 2013; competitive abilities: e.g. Strasser and Schwabl, 2004; Eising *et al.*, 2006; Partecke and Schwabl, 2008; Müller *et al.*, 2010; but see Vergauwen *et al.*, 2014; reproductive capacity: Uller *et al.*, 2005; Rubolini *et al.*, 2007; dispersal: Tschirren *et al.*, 2007; general activity, aggressiveness, boldness, or anti-predator behaviour: Strasser and Schwabl, 2004; Daisley *et al.*, 2005; Eising *et al.*, 2006; Tobler and Sandell, 2007; Partecke and Schwabl, 2008; Ruuskanen and Laaksonen, 2010; life span: Schwabl *et al.*, 2012), which also opens a window to potential trans-generational effects of maternal yolk hormones.

These long-lasting hormone-mediated maternal effects may differ between males and females (e.g. Tobler and Sandell, 2007; Ruuskanen and Laaksonen, 2010), with potentially opposing consequences for fitness-related traits. Thus, several studies have suggested that females may suffer negative effects on reproductive traits (reduced number of eggs: Uller *et al.*, 2005; reduced laying activity and egg fertility: Rubolini *et al.*, 2007; lower copulation frequency: Bonisoli-Alquati *et al.*, 2011a), whereas males may benefit from high yolk androgen concentrations via positive effects on traits that are potentially linked to male mating success such as sexual ornamentation (Eising *et al.*, 2006; Strasser and Schwabl, 2004; Bonisoli-Alquati *et al.*, 2011b; but see Rubolini *et al.*, 2006; Müller and Eens, 2009; Ruuskanen *et al.*, 2012a, 2013). On the other hand, if yolk androgens have a positive effect on offspring body size and condition at independence, this could indirectly have a positive impact on long-term fitness consequences that are size-dependent, such as the probability of acquiring a territory, recruitment or reproductive success. Thus, long-term studies could shed light on the fitness consequences of yolk hormones by measuring the effects of embryonic exposure to maternal androgens on overall survival, adult phenotype, behaviour and life-time reproductive success.

Considerations for interpreting the variable effects of yolk androgens

In experimental studies on hormone-mediated maternal effects, injections of androgens into the yolk are usually carried out to simulate a differential maternal allocation of resources in response to a hypothetical external stimulus. Thus, birds provide excellent models since their embryos develop outside the mother's body in relatively large eggs (Groothuis *et al.*, 2005a). Using injections of radiolabelled testosterone in freshly laid eggs, von Engelhardt and co-authors showed that the injected testosterone was metabolised in the egg from day 1 of incubation and taken up by the embryo (von Engelhardt *et al.*, 2009), suggesting that the embryo plays an active role in modifying the hormonal levels that mothers transfer to them. Experimental studies have demonstrated that maternal hormones, and in particular maternal androgens, can affect various aspects of offspring phenotype, including growth, physiology and behaviour (reviewed in Groothuis *et al.*, 2005a; Gil, 2008). Nevertheless, the results of previous studies on the effects of yolk androgens are not consistent either with respect to growth or to immune function. The exposure to elevated levels of maternal yolk androgens may in some cases reduce growth and survival of nestlings (Sockman and Schwabl, 2000, Rubolini *et al.*, 2006), or stimulate (Navara *et al.*, 2006) or not have any change in immune function (Tschirren *et al.*, 2005; Pitala *et al.*, 2009). In addition, several studies have found a positive relationship between yolk androgen levels and begging rate (Schwabl, 1996a, Eising and

Groothuis, 2003, von Engelhardt *et al.*, 2006, Barnett *et al.*, 2011) while other studies showed that androgen-treated birds were slower to respond and begged at lower rate (Pilz *et al.*, 2004) or simply no evidence of effects on begging (Boncoraglio *et al.*, 2006, Saino *et al.*, 2006, Müller *et al.*, 2010). Hence, the effects of yolk androgens on nestling growth, immune function, survival or behaviour are far from clear. Therefore, to draw more accurate conclusions about the effect of yolk androgens as modulators of the life-history trade-offs, we should consider that their consequences may vary depending on specific issues, such as species of study, sex, age, type of androgen, dose and environmental context.

Species-specific effects

Studies of a single species can do little to shed light on trade-offs between life-history traits (Stearns, 1992). Only comparative studies encompassing a large number of species can provide a thorough understanding of the selective forces that underlie the evolution of these traits (reviewed in Gil, 2003). As it has been previously mentioned, yolk androgen concentration may be strongly influenced by laying order, but this pattern changes completely depending on the species. Several studies have shown that androgen concentration increases with increasing laying order in Canaries, Red-winged blackbird or European starling (Schwabl, 1993a; Lipar *et al.*, 1999; Pilz *et al.*, 2003; respectively), while in other species, such as the Cattle egret or Zebra finch (Schwabl *et al.*, 1997, Gil *et al.*, 1999, respectively), the pattern is reversed. However, in other species there is no apparent trend (Whittingham and Schwabl, 2002; Ellis *et al.*, 2001). Thus, evidence for an adaptive value of androgens as regulators of sibling competition is not conclusive, since increasing levels of androgen with increasing laying order may buffer the effects of asynchronous hatching (Lipar and Ketterson, 2000; Eising *et al.*, 2001), but also have opposite effects (Sockman and Schwabl, 2000). Differences among species in the pattern of androgen allocation with respect to laying order imply the existence of different selective pressures (Gil, 2003). Differences between species are quite large, not only in concentration but also in the type of androgen (reviewed in Gil, 2003). In some species the most abundant egg androgen is androstenedione (Cattle Egret: Schwabl *et al.*, 1997), whereas in others it is testosterone (Common Canary: Schwabl, 1997a). Other studies suggest that the difference between studies may be a consequence of different feeding strategies between different species, or as a result of being precocial or altricial species (Andersson *et al.*, 2004; Groothuis and Schwabl, 2008).

Sex-specific effects

Maternal androgens can affect a given trait in both sexes or only in one sex (reviewed in Groothuis and Schwabl 2008; Gil, 2008). This suggests that elevated androgens in egg yolks may have differential effects on male and female embryos or offspring phenotype because of differential susceptibility of the two sexes to sex hormones (Badyaev, 2002). For example, in the American kestrel, androgen injections into yolk decreased growth of male but not female nestlings (Sockman *et al.*, 2008). Similarly, in zebra finches, experimentally elevated yolk androgen concentrations enhanced the growth rates of females but reduce them in males (Rutkowska and Cichon, 2006; Rutkowska *et al.*, 2007; von Engelhardt *et al.*, 2006), whereas in barn swallows, the effect was the opposite, females suffering while males benefiting from an increase in yolk androgens (Saino *et al.*, 2006). In addition, in the black-headed gulls, pharmacological treatment of eggs with an antagonist of the androgen receptor elevates growth of male nestlings and inhibits growth of female nestlings (Müller *et al.*, 2005a). These sexually contrasted effects of yolk androgens could have led to

mechanisms of sex-specific androgen exposure (e.g., Young and Badyaev, 2004). However, a constraint in the evolution of high levels of yolk androgens may also be related to antagonistic effects on male and female offspring (Müller *et al.*, 2005b, von Engelhardt *et al.*, 2006, Saino *et al.*, 2006, Rutkowska *et al.*, 2007), which could mean that mothers are not able to differentially allocate androgens in relation to embryo sex (Saino *et al.*, 2006). However, sex-specific effects are not always present (e.g., Eising *et al.*, 2006; Müller *et al.*, 2004). This heterogeneity could be due to differences in ontogenetic mechanisms relative to sexual dimorphism, but few data are available yet to conduct a comparative analysis of this aspect.

Type of androgen

Since the discovery of maternal androgens in avian egg yolk (Schwabl, 1993), a large number of studies on hormone-mediated maternal effects has emerged (reviewed in Groothuis and Schwabl 2008; Gil, 2008; Ruuskanen, 2015). However, the large difference among studies in the type of androgens injected hinders the task of drawing conclusions (**T+A4**: e.g. Sockman *et al.*, 2008; Pitala *et al.*, 2009; Ruuskanen *et al.*, 2012a, b; Eising and Groothuis, 2003; Müller *et al.*, 2005a, Müller and Eens, 2009; **A4**: e.g. Hegyi and Schwabl, 2010; Sockman and Schwabl, 2000; **T**: e.g. Lipar and ketterson, 2000; Andersson *et al.*, 2004; Tschirren *et al.*, 2007; Strasser and Schwabl, 2004; Schwabl *et al.*, 2011; Vergauwen *et al.*, 2011; Navara *et al.*, 2005; Tobler *et al.*, 2007, Barnett *et al.*, 2011). Gil and co-authors (2007) suggested that studies should consider at least the specific mechanisms of the 2 dominant androgens, A4 and T, and their differential metabolic pathways with respect to immunity and development, since the differential effect of yolk A4 on developmental periods and the contrasting influences of coloniality on the two androgens suggested that each androgen had specific actions. In this line, Hegyi and co-authors (2011) suggested that in some species, especially those with much more A4 than T in the yolk, A4 and not testosterone may be the yolk androgen with a long-term function and adaptive deposition pattern. According to these findings, a recent study reveals that yolk A4 and yolk T are associated with different biological consequences, since high yolk A4 concentrations lead to higher fitness, whereas high yolk T concentrations are associated with lower fitness in wild collared flycatcher (Tschirren *et al.*, 2014). In addition, A4 and T have different affinities for the androgen receptor (T > A4, reviewed in Goothuis and Schwabl, 2008). Moreover, A4 and T can induce different gene expression patterns via the androgen receptor pathway (Holterhus *et al.*, 2002). Consequently, yolk A4 and T can be expected to have different potencies in affecting developmental processes (reviewed in Goothuis and Schwabl, 2008). Further studies are required to show if selection is acting directly on A4 and T, their metabolites (e.g. estradiol), and/or the enzymatic pathways that mediate their conversion.

Age-specific effects

Yolk androgens of maternal origin not only exert effects on early stages of offspring life (e.g. Groothuis *et al.*, 2005a; Uller *et al.*, 2007; Navara and Mendonça, 2008), but also could lead to long-term effects into adulthood (Ruuskanen *et al.*, 2012; Ruuskanen *et al.*, 2013) since these maternal hormones may have organizational effects and long-lasting activational effects (reviewed in Carere and Balthazart, 2007; Groothuis and Schwabl, 2008). Beyond the short- and long-term consequences, several studies have found that yolk androgen effects may differ in smaller time scales, showing for example an interaction between androgen treatment and age on growth during the nestling stage (e.g. Schwabl, 1996a; Pilz *et al.*, 2004; Müller *et al.*, 2010; Hegyi and

Schwabl, 2010). In fact, it has been suggested that although egg components are important early in ontogeny, their effects quickly dissipate during early development, genetic and environmental having stronger effect thereafter (Smith and Bruun, 1998). This is consistent with comparative data by Schwabl and co-authors (2007) who found that the relationship between androgen levels and growth was stronger for the embryonic than for the nestling developmental period. In addition, other studies have shown that yolk androgen effects were stronger in the early days of nestling development (Schwabl, 1996a; Pilz *et al.*, 2004; Cucco *et al.*, 2008), which suggests that androgen sensitivity may be particularly high during early stages of development. These findings underline the importance of considering different phases of the juvenile period when studying the adaptive value of maternal androgens.

Dose-specific effects

In experimental studies, a crucial aspect to bear in mind is that the dose of androgen injections should be scaled to the natural variation of yolk androgen concentrations for a given species; otherwise the functional consequences of a possible pharmacological treatment may become difficult to interpret (reviewed in Groothuis *et al.*, 2005a). Even so, it is often assumed that, within the physiological range of yolk hormone concentrations of a species, increased dosages cause greater effects. However, there is no reason why this effect should necessarily fit to a linear reaction. Thus, dose–response curves could show, for example, a negative quadratic shape where intermediate dosages have greater effects than either higher or lower doses (reviewed in Groothuis and Schwabl, 2008). Most studies typically use one single dose, and the amount that is injected varies widely between studies, thus making it difficult to identify general patterns. Very few avian studies have examined whether the immunological consequences of yolk androgens vary depending on the injected concentration. In contrast to low doses of testosterone, high doses have inhibitory effects on the growth of the bursa of Fabricius in chick embryos, (Norton and Wira, 1977); which is a central humoral immune organ unique to birds that plays important roles in B cell development and antibody production (Mustonen *et al.*, 2001). In this vein, other studies have found that a super-physiological dose of testosterone resulted in detrimental effects on cell-mediated immunity in nestlings (Navara *et al.*, 2005; Cucco *et al.*, 2008). Thereby, a non-linear dose–response relationship might set an upper limit to hormone mediated maternal effects while at the same time might relax constraints between large amounts of a hormone for regulation of maternal functions and the resulting high exposure of the embryo (reviewed in Groothuis and Schwabl, 2008).

Context-specific effects

Maternal yolk androgen concentrations not only vary systematically across the laying sequence within clutches but also there is an even larger variation of maternal yolk androgen concentrations between clutches (Gil, 2003; Groothuis *et al.*, 2005a). A high between-clutch variation in androgen content of eggs suggests that the costs and benefits of yolk androgens may depend on environmental context (Pilata *et al.*, 2009). In this way, mothers may try to adjust the offspring phenotype for a given environment after hatching (Mousseau and Fox, 1998). This differential allocation of yolk androgens could be modulated by conditions experienced by the mother, such as food availability (Verboven *et al.*, 2003; Benowitz-Fredericks *et al.*, 2013), photoperiod (Schwabl, 1996b), aggressive interactions (Whittingham and Schwabl, 2002), the attractiveness of their mates (Gil *et al.*, 1999, 2004; Uller *et al.*, 2005), parasite abundance (Tschirren *et al.*, 2004; Postma *et al.*, 2014) or breeding density (Schwabl,

1997a; Groothuis and Schwabl, 2002; Pilz and Smith, 2004). Thus, for example, detrimental effects of yolk androgens may exceed beneficial effects when food is scarce, leading to a negative net effect on early growth, or carry the opposite effect when food is plentiful, leading to a positive net effect on early growth (Verboven *et al.*, 2003; Smiseth *et al.*, 2011). However, the context-dependent effect of yolk androgens on offspring development and physiology has been scarcely explored (Verboven *et al.*, 2003; Gasparini *et al.*, 2007; Benowitz-Fredericks *et al.*, 2013), and further works are needed to improve our understanding of the effect of environmental context in how maternal hormones affect life history trade-offs.

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OBJECTIVES

The general objective of this thesis is to study how maternally derived androgenic hormones regulate growth, immune function and life-history trade-offs in a wild bird population, and thereby set the stage for a complex benefit/costs dynamics of androgen allocation. To address this topic, we will use the spotless starling as a model species. Birds provide an excellent model to study yolk androgen effects since embryos develop outside the mother's body in relatively large eggs, facilitating experimental studies that are essential to understand the adaptive significance of patterns of variation in yolk androgen. The specific issues articulating this thesis are:

a. Given the ability to experimentally manipulate androgens levels in spotless starling eggs, it should be possible to tease apart the effects of the different androgens deposited by mothers in the egg yolks on offspring development. Although it is clear that maternal effects mediated by yolk androgens may alter offspring phenotype, there is a possible confounding effect due to large differences among studies in the type of androgens injected. **Chapter I** examines the differential effect on nestling developmental traits of two of the main yolk androgens, testosterone (T) and androstenedione (A4). For this purpose, eggs of spotless starlings were injected with physiological levels of either T, A4, a combination T + A4 or vehicle substance (control).

b. One important issue that has been overlooked in previous research is the likely non-linear nature (e.g. thresholds, maxima, etc.) of hormone effects on different offspring traits. By experimentally injecting three different androgen doses of the naturally occurring mixture of yolk T and A4 ('low', 'intermediate' and 'high' doses) into spotless starling egg yolks, in **Chapter II** we explore possible complex dose-response effects of maternal androgens on offspring development. Maternally derived androgens are generally believed to have a positive influence on offspring fitness, but it has been suggested that they could be balanced by some costs, mostly immunological. **Chapter II** focuses on the potential influence of yolk androgens on nestling growth, while **Chapter III** explores the physiological dose-response consequences on different branches of the immune system. This is a key issue since non-linear dose–response effects of yolk androgens may easily shift the balance from benefits to costs.

c. Differential allocation of resources among clutches may allow females to adjust the offspring phenotype to specific requirements of the environment. Such maternal modulation of yolk androgens as a function of the environmental conditions could be an adaptive strategy to handle the context- and dose-dependent effect of androgens. Hence, the balance between costs and benefits of androgen allocation is expected to depend on nestling environment. However, such hypothetical context-dependent effect of yolk androgens on offspring physiology has been scarcely explored. Hence **Chapter IV** examines the yolk androgens trade-offs between nestling growth and immune function taking into account the dif-

ferent breeding attempts across the same breeding season, as spotless starling is a multibrooded passerine.

d. Most previous experimental studies on yolk androgens have focused on their effects on the early stages of life, while there are relatively few experimental works on the long-term effects of maternal hormones. These long-lasting effects may arise from yolk androgens modifying certain physiological traits of the offspring. By monitoring nestlings from **Chapter IV** in the following three breeding seasons, in **Chapter V** we determine the long term consequences of the experimental manipulation of yolk androgens on adult phenotype, survival and breeding output.

The hatching time

The spotless starling breeds in tree holes and artificial cavities, showing high breeding synchrony. Females usually lay five immaculate blue-greenish eggs, whose colour can be used as a post-mating signal of quality. Egg incubation usually starts before the last egg is laid, and lasts for 12 days approximately.

This chapter is based upon the manuscript: **Muriel, J.**, Pérez-Rodríguez, L., Puerta, M. and Gil, D. (2013) Differential effects of yolk testosterone and androstenedione in embryo development and nestling growth in the spotless starling (*Sturnus unicolor*). *General and Comparative Endocrinology*, **194**, 175-182.

Differential effects of yolk testosterone and androstenedione in embryo development and nestling growth in the spotless starling (*Sturnus unicolor*)

Abstract: Yolk androgens in avian eggs play a significant role in embryo and nestling development. However, few studies have examined the differential effect of two of the main yolk androgens, testosterone (T) and androstenedione (A4). Here, we injected eggs of spotless starlings with physiological levels of either T, A4, the combination T+A4 or vehicle substance (control), to examine the differential ability of these steroids to influence nestling development. We found that the duration of the embryonic period was increased by T, and less so by A4, but not by the combination T+A4. Body condition was reduced in all experimental treatments where A4 was present, particularly so in the combination T+A4. Tarsus length was increased in males by A4, and in a lower degree by T, whereas the combination T+A4 inhibited growth. However, these differences in tarsus length between groups disappeared at the end of the nestling period. Cell-mediated immune responsiveness was marginally affected by the interaction between treatment and sex. These patterns suggest that in this species, T has a stronger influence during embryo development than A4, whereas during nestling development the capacities of both androgens to influence growth are similar. The combination T+A4 showed non-additive effects, suggesting either some kind of inhibition between the two androgens, or else an excessive effect due to a bell-shaped pattern of response. Our results suggest a complex picture of sex and age-dependent effects of T and A4, and underline the necessity of further research in the metabolism and action of egg androgens.

Keywords: Yolk androgens, nestling development**,** embryonic developmental period, testosterone, androstenedione, *Sturnus unicolor*.

Introduction

Since the discovery of testosterone of maternal origin in the yolk of birds (Schwabl, 1993), many studies have studied the effects of these hormones on development, growth, behavior and adult phenotypes in the eggs of several bird species (Groothuis *et al.*, 2005b; Gil, 2008). The initial excitement about the capacity of these hormones to boost growth (Winkler, 1993; Gil *et al.*, 1999), has weaned after the publication of several studies that have shown that the effects are less spectacular than previously expected. For instance, several experiments have found weak or non-existing effects of experimental injections of androgens (Rubolini *et al.*, 2006; Tobler *et al.*, 2007), complex interactions with sex (Saino *et al.*, 2006; von Engelhardt *et al.*, 2006) or effects that are dependent on environmental factors (López-Rull and Gil, 2009). Current opin-

The androgen injection

Mothers may adjust the development and the phenotype of their offspring to environmental conditions by an adaptive allocation of resources into the egg, such as nutrients, antioxidants, immunoglobulins, albumen lysozymes, and hormones. The avian egg is an excellent model to study maternal effects since their embryos develop outside the mother's body in eggs, and researchers can easily manipulate egg components by experimental injections.

This chapter is based upon the manuscript: **Muriel, J.**, Pérez-Rodríguez, L., Puerta, M., Gil, D. (2015) Diverse dose-response effects of yolk androgens on embryo de-velopment and nestling growth in a wild passerine. *The Journal of Experimental Biology*, **218**, 2241-2249.

Diverse dose-response effects of yolk androgens on embryo development and nestling growth in a wild passerine

Abstract: Avian egg yolks contain various amounts of maternally derived androgens that can modify offspring phenotype and adjust their development to posthatching environment. Seemingly adaptive variation in yolk androgen levels with respect to breeding density conditions or male attractiveness has been found in numerous studies. One important consideration that has been overlooked in previous research is the likely non-linear nature of hormone effects. To examine possible complex dose-response effects of maternal androgens on chick development, we experimentally administered three different androgen doses of the naturally-occurring mixture of yolk testosterone and androstenedione to spotless starling eggs (*Sturnus unicolor*). We found that yolk androgens show a non-linear dose-response pattern for several traits. Thus, androgens had a stimulatory effect on hatching body mass and nestling skeletal growth, but maximum values were found at intermediate doses, whereas our highest dose resulted in a decrease. However, the opposite *U-shaped* effect was found on nestling body mass. We also detected linear negative and positive effects on embryonic development period and nestling gape width, respectively. Our results suggest differential tissue responsiveness to yolk androgens, which may result in compromises in maternal allocation to produce adapted phenotypes. Due to the non-linear dose-response pattern, future investigations should carefully consider a wide range of concentrations, since the balance of costs and benefits may strongly differ depending on concentration.

Keywords: Yolk androgens, testosterone, androstenedione, maternal effects, *Sturnus unicolor*, dose-response.

Introduction

Females can influence the phenotype of their offspring through genes and somatic investments. Mousseau and Fox (1998) define maternal effects as epigenetic modifications of offspring phenotype caused by the environment provided by the mother during development. These mechanisms of phenotypic plasticity can cause evolutionary changes in some traits because they affect the expression of traits under selective pressures from heterogeneous environmental conditions (Mousseau and Fox, 1998; Price, 1998; Räsänen and Kruuk, 2007; Wolf and Wade, 2009).

Avian models are ideal prototypes for studying maternal effects in an evolutionary framework, since their embryos develop outside the mother's body in independent structures such as eggs. Egg production represents a substantial maternal investment for birds with a strong influence in offspring development and survival (Williams, 1994; Christians, 2002). Hormone concentrations in the yolk are considered a clear case of maternal effects

Appendix S1. Supplementary Methods

Accuracy of egg injections

We calculated the mean and standard deviation of injection volumes by performing mock injections within 0.2 ml eppendorf tubes and measuring the weight with a precision balance (A-2005, Sartorius Analytical Balance, Goettingen, Germany, accuracy = 0.0001 g) in the lab. The volume injected into the eggs was 10.15 ± 1.05 (mean ± SD) µl. For performing injections see further details in Muriel and co-authors (Muriel *et al.*, 2013). A pilot study was performed to check whether the medium containing the treatments actually reached the yolk. It consisted of injection of 10 µl of sesame oil stained with Neutral Red (an eurhodin dye used in histology, Winckler, 1974) into seven eggs from three non experimental nest-boxes and retrieved them after 5 days. Yolks were frozen and separated from the albumin. Yolks of all injected eggs contained a homogeneous amount of Neutral Red throughout (albumin contained none), suggesting that the treatments do diffuse uniformly into the yolk within the first days.

Sex determination

For sex determination, DNA was extracted from the blood samples using ammonium acetate techniques (Bensch and Åkesson, 2003), and diluted to a working DNA concentration of 25 ng/µl. This solution was used in a polymerase chain reaction (using the primers P2 and P8) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (Griffiths *et al.*, 1998). Amplified products were visualized in 1.5% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, CA). A subsample of 32 nestlings was run twice in several occasions to check error rate, and in all cases the sex determination was identical.

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Supplementary Table S3. Number of non hatched and hatched eggs per each treament.

Treatment	*Non Hatched*	*Hatched*	*Total*	*% Hatched*
Control	59	123	182	67.58
T2	13	43	56	76.78
T4	29	41	70	58.57
T8	24	45	69	65.21
Non injected	14	219	233	93.99

Supplementary Table S4. Initial mixed models for tarsus length, body mass, body condition and gape width of spotless starling on nestling period. Nonsignificant variables were removed from the model based on stepwise selection by *p-values* criterion ($P < 0.05$, corresponding to the numbers in bold).

	Tarsus length			**Body mass**			**Body condition**			**Gape width**		
Independent variable	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***
Treat	1/560	0.88	0.3496	1/566	0.02	0.8987	1/589	0.34	0.5623	1/591	10.83	**0.0011**
$Treat^2$	1/585	0.69	0.4061	1/584	0.73	0.3946	1/565	3.76	**0.0530**	1/560	0.22	0.6407
$Treat^2$ × Age [days]	3/538	4.94	**0.0022**	3/539	4.17	**0.0062**	3/545	1.68	0.1699	3/536	0.92	0.4297
$Treat^2$ × Sex	1/580	0.31	0.5796	1/582	0.62	0.4309	1/573	0.32	0.5736	1/580	0.76	0.3832
$Treat^2$ × Sex × Age [days]	3/530	0.13	0.9411	3/531	0.06	0.9796	3/532	0.40	0.7508	3/529	0.02	0.9962
Trt × Age [days]	3/538	2.30	0.0761	3/539	2.08	0.1020	3/548	1.45	0.2271	3/541	0.11	0.9540
Trt × Sex × Age [days]	3/533	0.20	0.8960	3/534	0.15	0.9307	3/535	0.43	0.7292	3/532	1.65	0.1760
Age [days]	3/538	3257.40	**<0.001**	3/539	1662.52	**<0.001**	3/551	0.00	0.9997	3/548	924.72	**<0.001**
Sex	1/575	10.99	**0.0010**	1/577	15.74	**<0.001**	1/588	0.56	0.4537	1/596	9.98	**0.0017**
Trt × Sex	1/573	0.52	0.4717	1/576	0.69	0.4050	1/587	0.20	0.6553	1/586	0.04	0.8420
Sex × Age [days]	3/538	5.68	**<0.001**	3/539	7.46	**<0.001**	3/539	0.01	0.9983	3/547	2.43	0.0640
Clutch laying date	1/130	26.65	**<0.001**	1/119	1.84	0.1771	1/89.6	13.96	**0.0003**	1/88.4	15.55	**<0.001**
Egg volume	1/261	5.41	**0.0208**	1/233	9.26	**0.0026**	1/140	9.77	**0.0022**	1/149	0.53	0.4674
Laying order	1/571	95.01	**<0.001**	1/574	90.77	**<.0001**	1/600	18.40	**<0.001**	1/592	1.38	0.2402
EDP	1/422	20.84	**<0.001**	1/418	13.11	**<0.001**	1/254	0.66	0.4171	1/250	3.97	**0.0474**
Brood size	1/582	3.65	**0.0566**	1/583	10.87	**0.0010**	1/591	1.17	0.2794	1/590	9.86	**0.0018**
Tarsus length	—	—	—	—	—	—	—	—	—	1/588	71.74	**<0.001**

Supplementary Table S5. Summary of final repeated-measures mixed models showing the estimates and standard error (estimate ± SE) of all the variables affecting tarsus length, body mass, body condition and gape width of nestlings. Models were run using Proc Mixed with Satterthwaite correction to adjust the degrees of freedom. Age was measured in days (3, 6, 10 and 14).

	Tarsus length	**Body mass**	**Body condition**	**Gape width**
Independent variable	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***
Intercept	29.958 ± 0.224	72.161 ± 0.979	-0.1610 ± 0.095	23.235 ± 0.139
Treat	0.156 ± 0.075	0.307 ± 0.335	-0.0175 ± 0.030	0.0829 ± 0.025
Age [3]	-18.709 ± 0.245	-58.865 ± 1.099	–	-3.645 ± 0.128
Age [6]	-11.520 ± 0.242	-39.906 ± 1.086	–	1.517 ± 0.128
Age [10]	-2.219 ± 0.242	-8.824 ± 1.086	–	2.713 ± 0.128
Treat × Age [3]	-0.212 ± 0.103	-0.585 ± 0.465	–	–
Treat × Age [6]	-0.214 ± 0.103	-0.775 ± 0.463	–	–
Treat × Age [10]	-0.0476 ± 0.103	0.224 ± 0.463	–	–
Sex (Males)	0.864 ± 0.185	4.571 ± 0.831	–	0.327 ± 0.104
Age [3] × Sex (Males)	-0.994 ± 0.252	-5.051 ± 1.130	–	–
Age [6] × Sex (Males)	-0.686 ± 0.250	-3.748 ± 1.124	–	–
Age [10] × Sex (Males)	-0.393 ± 0.250	-2.097 ± 1.124	–	–
$Treat^2$	-0.0916 ± 0.034	-0.0792 ± 0.152	0.0292 ± 0.015	–
$Treat^2$ × Age [3]	0.119 ± 0.045	0.309 ± 0.202	–	–
$Treat^2$ × Age [6]	0.148 ± 0.044	0.477 ± 0.200	–	–
$Treat^2$ × Age [10]	0.0313 ± 0.044	-0.161 ± 0.200	–	–
Clutch laying date	0.278 ± 0.054	–	-0.1210 ± 0.032	-0.177 ± 0.045
Egg volume	0.112 ± 0.048	0.643 ± 0.211	0.0959 ± 0.031	–
Laying order	-0.251 ± 0.026	-1.099 ± 0.115	-0.0823 ± 0.019	–
EDP	-0.184 ± 0.040	-0.644 ± 0.177	–	0.075 ± 0.037
Brood size	-0.0725 ± 0.038	-0.559 ± 0.169	–	0.117 ± 0.037
Tarsus length	–	–	–	0.258 ± 0.030

Parental care

Generally, female spotless starlings invest more than males in rearing the brood, although paternal care varies widely, with some pairs exhibiting biparental care and others in which females are left alone to care for nestlings. Feedings provided by parents can be divided among nestlings and consists mainly of insects. Fledglings leave the nest around 22 days of age.

This chapter is based upon the manuscript: **Muriel, J.**, Pérez-Rodríguez, L., Ortiz-Santaliestra, M. E., Puerta, M., Gil, D. Sex-specific effects of high yolk androgen levels on constitutive and cell-mediated immune responses in a wild passerine. *Manuscript*.

Sex-specific effects of high yolk androgen levels on constitutive and cell-mediated immune responses in nestlings of an altricial passerine

Abstract: Avian embryos are exposed to yolk androgens that are incorporated into the egg by the ovulating female. These steroids can affect several aspects of embryo development, often resulting in increases in overall size, or in the speed of growth of different traits. However, several studies suggest that they also entail immune costs to the offspring. In this study we explored whether variation in yolk androgen concentration affected several measures of the constitutive and cell-mediated immune axes in the spotless starling (*Sturnus unicolor*). Using a within-brood design, we injected different doses of androgens (testosterone and androstenedione) in the eggs. Our study showed that experimentally increased yolk androgens led to sex-specific immunosuppression of two measures of innate and adaptive axes of the immune system. Both cell-mediated immune response and lysozyme activity decreased with increasing androgen levels injected in the egg in the case of male nestlings, whereas there were no effects on females. We found no effects of the experimental treatment in hemolysis or agglutination capacity, but these measures were negatively correlated to cell-mediated response, suggesting negative covariance among different branches of the immune system. Our results show that in our study species, yolk androgens induce immunosuppression in some axes of the immune system. Further studies should analyse the proximate causes for these contrasting effects in different axes of the immune system and the reason for the differential impact on males and females.

Keywords: Yolk hormones, testosterone, immunocompetence, parasites, androstenedione, maternal effects, *Sturnus unicolor*

Introduction

Avian yolks contain important amounts of several androgens that are produced by female birds during ovulation (Gil, 2008; Groothuis *et al.*, 2005b). Yolk androgens have been shown to affect several aspects of embryo development and lead to both short- and long-term effects on various offspring traits (e.g. Partecke and Schwabl, 2008; Strasser and Schwabl, 2004). Although the effects vary among species, many studies suggest that during embryo and nestling development, maternal androgens induce increases in overall size, or in the speed of growth of different traits (e.g. Navara *et al.*, 2006; Pilz *et al.*, 2004). These increases in growth can result in a modification of within-nest hierarchies among nestlings (Eising *et al.*, 2001; Müller *et al.*, 2004), or in overall

differences between broods in growth and survival (Müller *et al.*, 2007).

Given these apparent benefits, the occurrence of important levels of variation within and among clutches in androgen content suggests that androgen deposition may also entail costs for mothers or offspring (Gil *et al.*, 1999). Several possibilities for constraints have been proposed, including mismatches between parental care and nestling demands (Hinde *et al.*, 2010), negative effects in female fertility (Rutkowska *et al.*, 2005), sexually antagonistic effects (Saino *et al.*, 2006) or pleiotropic effects limiting optimal allocation for a given trait (Gil, 2008). However, the hypothesis that has received the largest attention in the literature so far is the immunocompetence handicap hypothesis (Folstad and Karter, 1992), which is based on the negative effects that androgens cause in the immune system. This hypothesis was initially proposed to account for possible costs limiting the development of exaggerated sexually selected traits. The evidence for the immunocompetence handicap hypothesis is mixed, depending on the group of taxa that is examined (Roberts *et al.*, 2004), but several studies suggest that immune costs, either through a direct or an indirect pathway, can play a role in the balance of costs and benefits of androgens (Owen-Ashley *et al.*, 2004).

In the case of yolk androgens, several lines of evidence suggest that immune costs might limit the amount of androgens that bird eggs contain. For instance, cell-mediated and humoral immunity were found to be reduced in black-headed gull chicks (*Larus ridibundus*) which had been exposed to high levels of androgens in the egg (Groothuis *et al.*, 2005a; Müller *et al.*, 2005). A similar reduction in both branches of the immune system was found in jackdaws (*Corvus monedula*) (Sandell *et al.*, 2009). In the same line, experimentally infected house martins (*Delichon urbica*) and great tits (*Parus major*) laid eggs with lower androgen levels than control birds (Gil *et al.*, 2006; Tschirren *et al.*, 2004), although a recent study failed to find an effect following a similar experimental approach (Heylen *et al.*, 2012). However, further studies in other species have found an enhanced immune response to experimentally increased androgens in eggs (Navara *et al.*, 2006), no effects (e.g. Andersson *et al.*, 2004; Pitala *et al.*, 2009; Rubolini *et al.*, 2006a; Tschirren *et al.*, 2005), or else have uncovered the role of additional factors that affect this pattern. For instance, in zebra finches, Rutkowska and coauthors found that whereas experimental male nestlings suffered a reduction in T-cell response, the pattern was inversed for female chicks (Rutkowska *et al.*, 2007). Also, in a previous study by our team we found that immune responses could actually be enhanced by yolk androgen injections, in this case in first broods of spotless starlings, presumably because of high resource availability (Muriel *et al.*, 2015b).

Several studies have suggested that the trade-off between immunity and yolk androgens could arise by a variety of mechanisms, including the specific inhibition of immunity by androgens, based for instance on the presence of androgen receptors (AR) in some immune cells (Ahmadi and McCruden, 2006; Gil and Culver, 2011), or through the activation of a corticosteroid route to immune inhibition (Owen-Ashley *et al.*, 2004). Regardless of the mechanism, one expectation is that there should be a trade-off between growth and immunity, and this should be open to empirical testing.

On the other hand, the immunological system is a set of several defense mechanisms composed of several axes, including innate and adaptive immunity, and comprising cell-mediated and humoral responses. Therefore, it is not possible to measure overall strength and efficacy of the immune system with a single immune assay (Adamo, 2004). In addition, previous studies have shown that correlations

among immune responses need not be positive, and that individuals may trade-off different aspects of their immunocompetence (Forsman *et al.*, 2008; Palacios *et al.*, 2012; Salvante, 2006). Trade-offs could be a consequence of an imbalance in the distribution of resources since adaptive immune responses are physiologically more costly than innate responses (Palacios *et al.*, 2009). Thus, any variation in maternally-derived components, such as androgens, may mediate increased investment in one branch of immunity at the expense of another. For example, Clairardin and co-authors (2011) found that increased *in ovo* testosterone induced a trade-off between bactericidal activity and cutaneous immune response in house wrens (*Troglodytes aedon*). In a similar study conducted in zebra finches (*Taeniopygia guttata*), elevation of egg testosterone levels also resulted in long-lasting positive effects on humoral, but not cell-mediated immune function (Tobler *et al.*, 2010).

One problematic aspect in the study of yolk androgens is the variation in the injected doses in the different experiments conducted so far (Muriel *et al.*, 2015a). This variation is likely to be a major cause of the diversity of effects found across studies, and it limits the general inferences that we can draw. Experimental dosage is an important issue, because steroids are known to present non-linear, dose-response effects (Navara *et al.*, 2005). We have previously shown (Muriel *et al.*, 2015a) a variety of dose-response patterns, depending on the morphological trait that is studied, in the spotless starling (*Sturnus unicolor*). In this study, we used a similar approach to inquire about the effects of an increasing amount of yolk androgens in a suite of immune responses and health status in the same species. We explored several components of the immune response, including cell-mediated immune response, hematological parameters and several measures of constitutive immunity, including antibacterial capacity of plasma and lysozyme activity. Since immune deficiency is expected to lead to a reduction in the capacity of the organism to withstand attacks by parasites, we also analyzed differences in the community of blood (trypanosome and haemosporidians) and intestinal (coccidia) parasites.

Material and Methods

Study area and species

The experiment was conducted in a large nest-box colony of spotless starlings (*Sturnus unicolor*) located near Madrid (Soto del Real). Yolk hormone manipulations were conducted between mid-April and mid-May 2010. The study area is a mixed woodland, used for cattle grazing, and mostly composed of oak (*Quercus pyrenaica*) and ash (*Fraxinus angustifolius*). The spotless starling is a relatively long lived, colonial and sedentary passerine species that exhibits a facultatively polygynous breeding system (Moreno *et al.*, 1999, Veiga, 2002). This omnivorous songbird (Peris, 1980) is sexually dimorphic, males being larger than females (Hiraldo and Herrera, 1974). Incubation usually starts before the last egg is laid (3-6 eggs per clutch), and it is done mainly by females (lasts for 12 days approximately) although parental care is provided by both pair members (Moreno *et al.*, 1999). The nestling period lasts about 21-22 days (Cramp, 1998; Fig. 12). Females can lay up to two clutches per season; the first one in early April and the second one about the end of May in our study area (López-Rull *et al.*, 2011). A replacement clutch could be laid as a result of the loss of the first clutch by predation or intraspecific competition (Müller *et al.*, 2007; Muriel *et al.*, 2015a). In this study we used chicks belonging to first (from 15th April to 19th May) and replacement broods (from 9th May to 13th June). The daily average

Figure 12. Spotless starling fledgling about 25 days old. *Picture credit: Jaime Muriel.*

maximum temperature and precipitation (mean ± SE) recorded per each breeding attempt for the year of study were 16.82 ± 0.77 °C and 2.35 ± 0.84 L m^{-2} for the first brood, and 21.07 ± 0.99 °C and 1.79 ± 0.60 L m^{-2} for replacement broods (data provided by the Spanish Meteorological Agency, AEMET).

Egg Injections

From the end of March onward, nest-boxes were inspected each day to determine laying date and order. Eggs were marked with a waterproof marker as they were laid. Injections began when the third egg was found in the nest, and the following eggs were injected the same day they were laid. Clutches were randomly assigned to one of three experimental treatments. Within each clutch, eggs were alternatively injected with either control or experimental injections, following laying order. The sequence of injections (i.e. starting with experimental or control) was modified between consecutive nests.

Experimental injections consisted of a combination of androgens dissolved in 10 µl of sesame oil (ref. 85067, Sigma-Aldrich, Steinheim, Germany). Control eggs received an injection of sesame oil only (10 µl). Experimental eggs received one of the following treatments: (1) low androgen dose consisting of 12 ng testosterone (T) and 34 ng androstenedione (A4) (refs. 86500 and A9630 respectively, Sigma-Aldrich, Steinheim, Germany), (2) intermediate androgen dose: 24 ng T + 68 ng A4, or (3) high androgen dose: 48 ng T + 136 ng A4. The low, intermediate and high doses were equivalent to, respectively, 2, 4 and 8 SDs of the population means for an average 1.4 g egg (testosterone: 14 ng/yolk [SD = 6.0], androstenedione: 50 ng/yolk [SD = 17.1], Müller *et al.* 2007)).

In ovo injections were performed in the field using a standard U-50 insulin syringe (Terumo Corporation, Tokyo, Japan), following a standard protocol described elsewhere (Muriel *et al.*, 2013; Muriel *et al.*, 2015a).

The experiment was carried out in 88 clutches, but we could only use 41 of them due to predation, destruction by other females or impossibility to assign hatchlings to their experimental group (this is not an unusual rate of nest failure in this population: Müller *et al.*, 2007). We included in the analysis data from 153 chicks (75 males and 78 females), although sample size for the different tests differs because for some individuals we were not able to collect enough blood volume to perform all the immunological assays.

Nestling data and sampling

Broods were visited frequently around the predicted hatching date (10-11 days after the last egg was laid) to be able to detect nestlings as they hatched and assign them to their specific experimental group (chicks were labelled by subtle cuttings in their dawn).

Nestlings were measured on days 3, 6, 10, and 14 post-hatching, in order to record growing patterns for a parallel study (Muriel *et al.*, 2015a). In the present analysis we will only use data from day 14. Body mass was recorded with a digital balance (Ohaus Scout II SC2020, China, accuracy = 0.1 g), and tarsus length with digital callipers (Mitutoyo Absolute, Japan, accuracy = 0.01 mm). All measurements were performed by the same person (JM) and blindly to individual treatment whenever possible.

After measuring T-cell-mediated immune response (see below), we took a blood sample (600 µL) of each nestling on day 15 posthatch. This sample was extracted from the jugular vein with heparinized syringes in order to carry out immunological tests and molecular sexing. Samples were kept on an ice box until arrival at the lab, and plasma was separated from the cell pellet by centrifuging at 5000 g and 4ºC for 10 minutes. Plasma was stored in two separate aliquots at -80° until analysis. Also, faecal sample were collected from 142 of 153 nestlings while they were handled, kept in ice until arrival at the lab and stored at -80° until analysis. In order to determine the presence of coccidian infections (Watve and Sukumar, 1995), fecal samples were collected in the afternoon (17 to 21 PM), since previous studies have suggested that oocyst discharge is much greater in the afternoon than in the morning (Brown *et al.*, 2001; Dolnik, 1999).

DNA extraction and molecular sexing

DNA extraction from blood samples was performed using an ammonium acetate method (Bensch and Åkesson, 2003), and diluted to a working DNA concentration of 25 ng/µl. Sex determination was carried out by amplifying (through polymerase chain reaction - PCR) an intron of the CHD1 genes on the avian sex chromosomes (Griffiths *et al.*, 1998). PCR products were electrophoresed for 60–90 min at 100 V in 1.5% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, CA) and were visualized under UV light, where one band was scored as male and two bands as female. In order to assure accurate assignment of the sex, the DNA extraction and PCR was carried out twice for 32 of 153 samples. In all cases the sex determination was identical.

Cell-mediated immune response

We evaluated T-cell-mediated immune response (CMI) on 14 days-old nestlings, using a dermal phytohaemagglutinin (PHA) reaction in the wing web, following a standard protocol (Smits *et al.*, 1999). After taking three measures of thickness of a plucked area of the left wing web with a thickness gauge (Mitutoyo Co., Tokyo, Japan) to the nearest 0.01 mm, we injected subcutaneously in that point 0.05 ml of a 5 mg/ml solution of PHA (L-8754, Sigma–Aldrich Chemie, Steinheim, Germany). After 24 ± 1.3 h, we took 3 new measurements of the

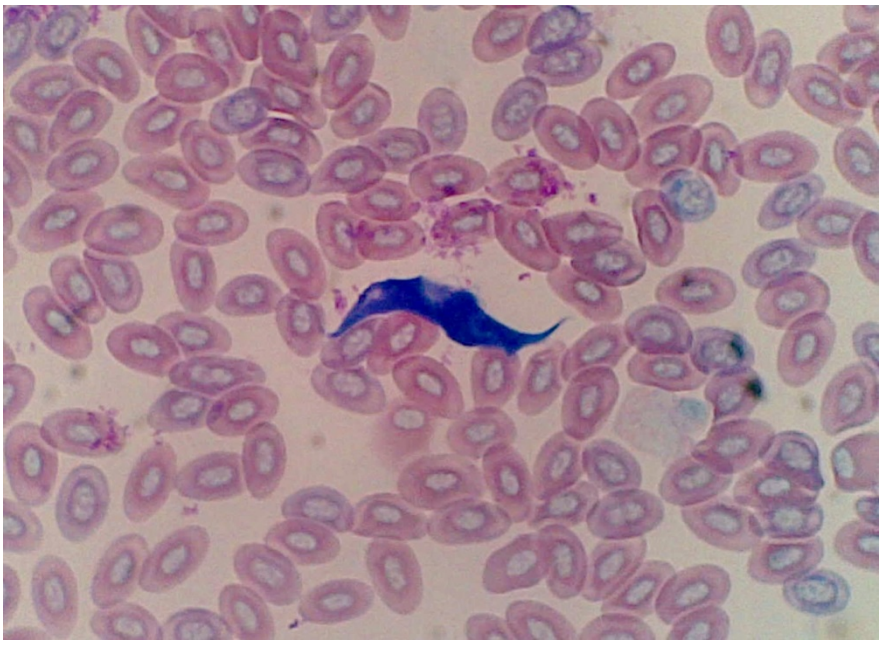

Figure 13. Hematozoic trypomastigotes of *Trypanosoma sp*. from a spotless starling nestling at day 14 posthatch. Giemsa-stained thin blood films. *Picture credit: Jaime Muriel.*

thickness of left wing web at the same point. As the repeatability of the measurements is high (Smits *et al.*, 1999), we used the mean for statistical analysis. Cell-mediated immunocompetence (CMI) was estimated as the difference between initial and final meas-urements of the left wing web swelling (Smits *et al.*, 1999). All measurements were done by the same individual (JM), blind with respect to treatment.

Leukocyte and hemoparasite counts

For identification of blood parasites (Fig. 13) and leucocytes, a drop of blood was smeared on one individually marked microscope slide. Once the blood had air dried, we fixed the slide by 3 min immersion in 100 % methanol and stained it using commercial Giemsa diluted with PBS pH 6.8 (1:2). Slides were examined under the microscope using the oil immersion objective (1000× magnification) to estimate the proportion of different types of leucocytes and hemoparasites (Campbell and Ellis, 2007; Merino *et al.*, 2001). Estimates of the total white blood cell count (WBC) and intensity of infection were calculated per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with respect to WBC, which were classified as heterophils, eosinophils, basophils, lymphocytes or monocytes. We also took the ratio of heterophils/ lymphocytes (H/L ratio) and the total leukocyte count as a measure of physiological stress and immunity, respectively, in birds (Gross and Siegel 1983; Maxwell and Robertson 1998). By screening smears, we could only identify intra- or extra-erythrocytic hemoparasites to genus level. One person (JM) conducted all cell and parasite counts to eliminate variation between observers.

Coccidia abundance

Protozoan coccidia are one of the most common intestinal parasites in birds (Svobodova *et al.*, 2015; Zinke *et al.*, 2004). Quantitative analysis of coccidian oocysts found in each fecal sample was carried out using a flotation technique (Villanua *et al.*, 2006). Fecal samples were extracted from collection tubes and extended in filter paper for 5 minutes to remove formal-

dehyde remnants. After that, approximately 0.5 g of feces were homogenized and suspended in 5 ml of a saturated $ZnSO_4$ solution (specific density 1.18). Oocyst counts were performed using a MacMaster chamber and their concentration (oocysts per gram of feces) calculated taking into account the exact weight (to the nearest 0.001 g) of each sample. Based on oocyst morphology (four sporozoites within each of two sporocysts), coccidia detected were identified as *Isospora* spp., a protozoan gut parasite belonging to the *Eimeria* complex. Because of its direct life cycle, avian *Isospora* species require no vector for the spread of infection, and transmission occurs if an appropriate host ingests sporulated oocysts (Fayer and Reid, 1982). We did not detect other intestinal parasite propagules in the samples analyzed.

Hemolysis-hemagluttination assays

Levels of natural antibodies and complement were quantified following Matson *et al.* (2005). We prepared twofold serial dilutions of plasma into phosphate buffered saline (PBS) across a 96-well plate using one row per bird (12 wells). Each row contained plasma at dilutions ranging from 1 through 1/1.024 in volumes of 25µl. We added to each well 25 µl of 0.1% sheep red blood cell suspension prepared with blood extracted from a sheep housed at the Dehesa de Galiana farm (Ciudad Real, Spain) less than 48 h before the assay and stored refrigerated until processing . Plates were covered and incubated at 37ºC for 60 min, tilted at a 45° angle for an additional 60 min, and then scanned under a stereomicroscope. We scored each sample by taking the value of the lowest plasma dilution that was sufficient to induce hemagglutination and hemolysis. Therefore, higher scores reflect, respectively, higher levels of natural antibodies and higher combined activity of the complement with natural antibodies. All scores were performed by the same person (MEO-S) who was blind to the identity of each sample. Repeatabilities, for a subset of 31 random samples assayed in duplicate, were high both for hemolysis (r = 0.86, $F_{30,31}$= 13.8, $P < 0.001$) and hemagglutination (r = 0.91, $F_{30,31}$ = 20.1, $P < 0.001$).

Lysozyme activity

Lysozyme is one the main antimicrobial proteins of the blood, being produced by most types of leukocytes (Gill, 1995). To measure lysozyme activity of plasma samples we used a 600 mg/l suspension of *Micrococcus lysodeikticus* (ref. M3770-5G, Sigma-Aldrich, Steinheim, Germany) in PBS. In each well of a 96-well plate, we added 200 µl of this bacterial suspension to 20 µl plasma or PBS wells, which were used as blanks. The activity of lysozyme is proportional to the rate of absorbance reduction due to the lysis *M. lysodeikticus* present in the suspension. To quantify this process, plates were incubated at 37°C and we measured absorbance at 850 nm at 15, 30, 45 and 60 min using a microplate reader (Biotek Powerwave XS2, Winooski, VT, USA). Plasma samples were assayed in duplicate. For each sample, we calculated the regression slope between absorbance (after subtracting the absorbance of blanks) and time. In order to quantify lysozyme concentrations (in µg/ml), a standard curve elaborated by serially diluting crystallized lysozyme (ref. L-6876, Sigma-Aldrich, Steinheim, Germany) in PBS was also included in all plates and treated in the same way as plasma samples. The repeatability of lysozyme quantification was high (r = 0.90, $F_{48,49}$ = 19.9, $P < 0.001$).

Statistical Analyses

All calculations were performed in the r language v. 3.1.0 (R Core Team, 2015). We analysed the data applying general lineal mixed models with the *lmer* function in the 'lme4' package (Bates *et al.*, 2014), and depending on the data distribution we used Gaussian, Poisson or binom-

Table 4. Results of a mixed linear model testing the predictors of nestling cell-mediated immune response (swelling response), where nest is declared as random effect. χ^2 (df = 1) and *P* values arise from a type III analysis of deviance on the model. In the case of factors, estimates refer to the second level of each group (females in the case of sex, and replacement broods in the case of brood). Sample size is 153 individuals.

Term	χ^2	*P*	Estimate (SE)
Treatment	1.01	0.317	-0.031 (0.03)
Sex	4.54	0.033	-0.375 (0.17)
Pre-swelling	0.34	0.555	0.046 (0.08)
Brood	4.30	0.038	-0.393 (0.19)
Body mass	2.10	0.147	-0.245 (0.17)
Growth	7.42	0.006	0.497 (0.17)
Treatment*Sex	4.91	0.027	0.095 (0.04)

Table 5. Results of a mixed linear model testing the predictors of nestling lysozyme activity, where nest is declared as random effect. χ^2 (df = 1) and *P* values arise from a type III analysis of deviance on the model. In the case of factors, estimates refer to the second level of each group (females in the case of sex, and replacement broods in the case of brood). Sample size is 93 individuals.

Term	χ^2	*P*	Estimate (SE)
Treatment	3.17	0.074	-0.089 (0.05)
Sex	0.76	0.381	-0.234 (0.27)
Brood	2.63	0.104	-0.346 (0.21)
Body mass	0.78	0.375	-0.185 (0.21)
Growth	0.04	0.833	-0.044 (0.21)
Treatment*Sex	6.29	0.012	0.182 (0.07)

ial negative distributions. Experimental treatment and sex were the fixed factors (predictor variables). Treatment was treated as continuous rather than categorical because androgen doses increased stepwise (Muriel *et al.*, 2015a). Nest was defined as a random effect affecting the model intercept. Information criteria (lowest AIC value) were used to select the best models. All biologically meaningful double interactions were included in the original model.

Results

Cell-mediated immune response

The best supported model contained a positive effect of growth and an interaction between treatment and sex indicating that immune response was differentially affected by treatment in males and females (Table 4). The significant interaction resulted from negative and positive trends for males and females, respectively (Fig. 14). However, separate GLMs for males and females failed to find a significant pattern of treatment in either sex (males: χ^2 = 2.07, *P* = 0.15, estimate (SE) = -0.045 (0.03); females: χ^2 = 2.86; *P* = 0.09, estimate (SE) = 0.051 (0.03). Furthermore, after controlling for body size, birds that grew more also had stronger immune responses (Fig. 15). Irrespective of treatment, cell-mediated immune response showed a seasonal decline in replacement broods and lower responses in females (Table 4).

Constitutive immunity measurements

Hemolysis and agglutination: Lysis levels

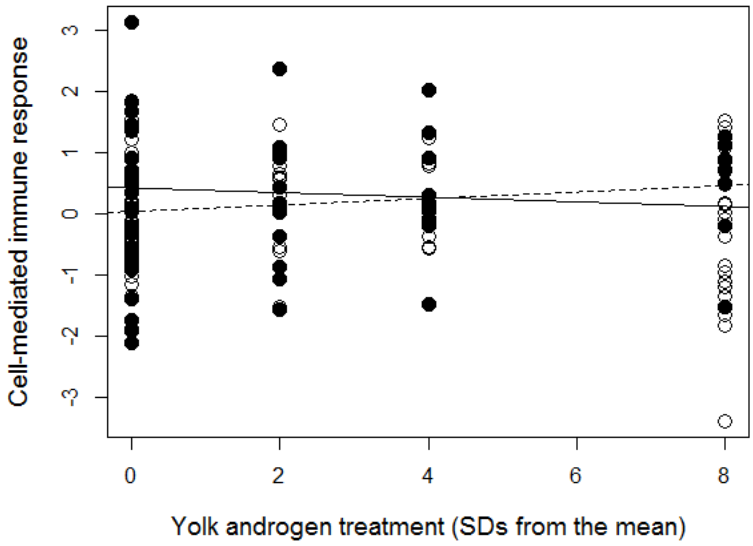

Figure 14. Effects of yolk androgen treatment on cell-mediated immune response (swelling of the wing web in response to PHA injection). The continuous line represents the estimate for males and the dotted line for females.

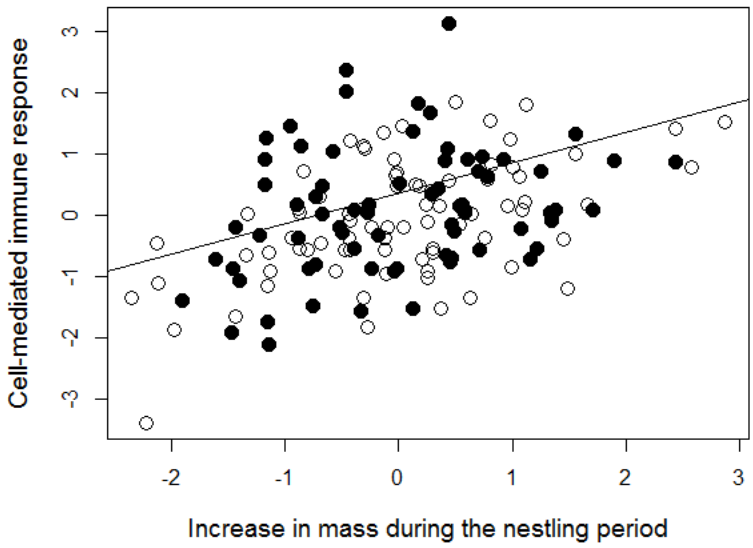

Figure 15. Relationship between cell-mediated immune response (swelling of the wing web in response to PHA injection) and growth during the nestling stage.

were not affected by treatment, sex, body mass or growth (all $P > 0.23$), but strongly increased in replacement with respect to first broods ($\chi^2 = 12.32$, $P < 0.001$; estimate (SE) for the replacement brood: 0.72 (0.20). Agglutination was similarly dependent on brood ($\chi^2 = 9.18$, $P < 0.01$; estimate (SE) for the replacement brood:

Table 6. Correlation among the four immunological measurements obtained. Sample size is 94 individuals and asterisks indicate: * P <0.05, *** P < 0.001.

	Haemolysis	Agglutination	Lysozyme activity
Cell-mediated immune response	-0.145	-0.206*	0.002
Haemolysis		0.765***	-0.016
Agglutination			-0.041

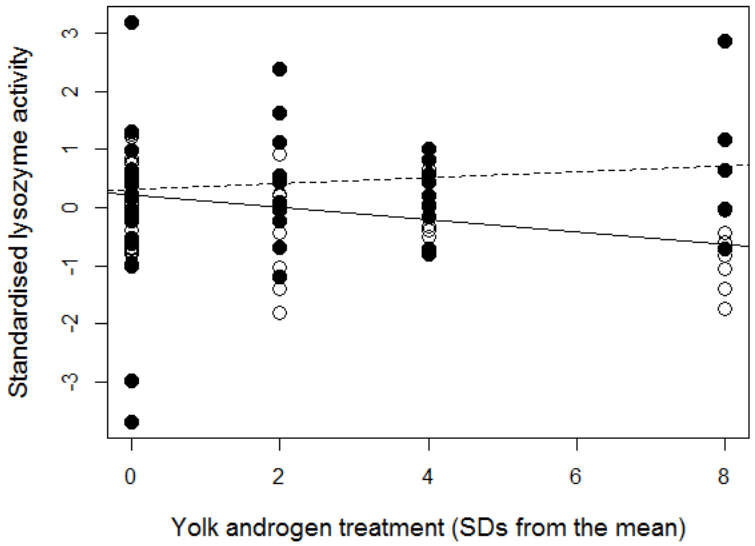

Figure 16. Effects of yolk androgen treatment on lysozyme antibacterial activity. The continuous line represents the estimate for males and the dotted line for females.

0.65 (0.21), increased with increasing body mass (χ^2 = 6.32, P < 0.02; estimate (SE): 0.24 (0.09), and was not affected by treatment, sex or growth (P > 0.18).

Lysozyme activity in plasma: The GLM for lysozyme activity showed a significant interaction between treatment and sex, with males displaying lower levels of lysozyme activity with increasing yolk androgen dose and no effect on females (Table 5; Fig. 16), and neither brood, body mass or growth were significant. Separate models for males and females confirmed this interaction, showing a highly significant negative effect of treatment on males (χ^2 = 6.56, P < 0.01, estimate (SE): -0.09 (0.03), and a non-significant positive trend for females (χ^2 = 2.24, P = 0.14, estimate (SE): 0.10 (0.069).

Correlations among immunological measurements

We ran Pearson correlations on the normalised scores of the four measurements of immunity that we obtained (Table 6). The data show that agglutination and hemolysis scores are strongly correlated among them. Cell-mediated immunity is however negatively correlated with agglutination, and shows a similar trend with respect to hemolysis. Lysozyme activity stands out as an independent component of the immune system unrelated to the rest (Table 6).

Table 7. Results of models testing the predictors of several haematological parameters, where nest is declared as random effect. Models are either GLMs on normalised values when possible, or else GGLMs with Poisson error distributions. χ^2 (df = 1) and *P* values arise from a type III analysis of deviance on the model. In the case of factors, estimates refer to the second level of each group (females in the case of sex, and replacement broods in the case of brood). Sample size is 153 individuals.

White blood cell type	Terms	χ^2	estimate (SE)	*P*
Heterophil/lymphocyte ratio	Treatment	1.41	0.03 (0.02)	0.234
	Sex	0.01	-0.01 (0.15)	0.975
	Brood	8.64	-0.54 (0.18)	0.003
	Body mass	9.30	-0.27 (0.08)	0.002
% eosinophils/total leukocytes	Treatment	0.20	0.01 (0.02)	0.653
	Sex	0.05	0.04 (0.15)	0.809
	Brood	1.70	0.26 (0.19)	0.192
	Body mass	6.60	0.24 (0.09)	0.010
% basophils/total leukocytes	Treatment	3.24	-0.03 (0.02)	0.071
	Sex	0.87	-0.12 (0.12)	0.350
	Brood	45.2	-0.90 (0.13)	<0.001
	Body mass	0.47	-0.05 (0.07)	0.488
% monocytes/total leukocytes	Treatment	0.01	-0.01 (0.02)	0.989
	Sex	1.03	-0.15 (0.15)	0.310
	Brood	36.75	-0.97 (0.16)	<0.001
	Body mass	0.89	-0.08 (0.08)	0.345
% total leucocytes/erythrocytes	Treatment	2.42	-0.04 (0.03)	0.119
	Sex	3.10	0.28 (0.16)	0.078
	Brood	0.03	0.03 (0.19)	0.853
	Body mass	0.13	0.10 (0.09)	0.256

Haematological parameters

We ran individual models for percentages of each leukocyte type over total leukocytes, for total number of leukocytes and for the ratio of heterophil to lymphocyte types (Table 7), testing the predictors, and respecting the specific error distribution of each variable. Treatment was not related to any parameter, although there was a non-significant tendency ($P = 0.07$) for basophils to decrease with increasing yolk androgen dose. There were no significant differences between sexes in the composition of blood cell types. Eosinophils increased with body mass, whereas the heterophil/lymphocyte ratio decreased. Replacement broods were characterised by significantly lower levels of basophils and monocytes and a reduction of the heterophil/lymphocyte ratio.

Parasites

We detected no malaria parasites (*Haemoproteus*, *Plasmodium* or *Leucocytozoon*) in the first 50 blood smears that we analysed, and thus we stopped checking for them in the rest of them. However, *Trypanosoma* spp. was found with a moderate prevalence (19.73%, N =152). A binomial GLM showed that the presence of *Trypanosoma* in blood was not related to treatment, sex or body mass (all estimates $P > 0.37$), but strongly increased from first to replacement broods ($F = 11.23$, $P < 0.001$; estimate (SE) for the replacement brood: 2.59 (0.77).

Using a GLMM with negative binomial distribution we found that the number of coccidia in faeces was also not related to treatment, sex or body mass (all $P > 0.10$), but strongly increased from first to replacement broods ($\chi^2 = 9.57$, $P < 0.01$; estimate (SE) for the replacement brood: 1.64 (0.51).

Discussion

Our study examined how several aspects of nestling innate immunity were modified as a response to an experimental increase in yolk androgen levels. The general pattern indicates that both sexes react different in this species, males being more immunologically compromised by yolk androgens than females (see below). There was no general effect across sexes, even though our treatment encompassed a wide range of yolk androgen doses, and was designed to test for possible dose-dependent effects. There were sex-specific patterns for cell-mediated immune response and lysozyme activity, but neither innate lysis or agglutination efficiency, or general immune condition as measured by haematological parameters showed immune suppression. In agreement with these patterns, neither haematozoan parasites nor coccidial infection were increased as a result of the yolk androgen manipulation.

We detected two sex-specific patterns, in which both male plasma lysozyme activity and cell-mediated immune response decreased with increasing yolk androgen levels. These results are consistent with a previous study conducted in the same starling population, where male chicks that hatched from yolk androgen-treated eggs suffered a slight suppression of their cell-mediated immune responsiveness (Muriel *et al.*, 2013). A similar pattern has been previously found in the zebra finch *Taenyiopigya guttata* (Rutkowska *et al.* 2007), which matches with the sex-specific effects of yolk androgens on growth in that species (von Engelhardt *et al.*, 2006).

Evidence from previous studies shows that yolk androgen manipulations can affect the growth of males and females differently in some species (Saino *et al.*, 2006; von Engelhardt *et al.*, 2006), and a recent review concluded that these differential effects tend to happen more often in species with strong sexual size dimorphism (Tschirren, 2015). However, in our previous studies in the spotless starling, we have not found sex-specific effects of yolk androgens on nestling growth (Müller *et al.*, 2007; Muriel *et al.*, 2013; Muriel *et al.*, 2015a; Muriel *et al.*, 2015b). In a parallel study using the same experimental setup employed here we found that elevated yolk testosterone resulted in enhanced somatic growth in both sexes (Muriel *et al.*, 2015a). Our data suggest that this differential effect of sex is independent of absolute size, and that is rooted in sex-specific processes so that only males, the larger sex, pay an extra immunological cost, as often shown in several studies across a diverse range of species (Fargallo *et al.*, 2002; Lobato *et al.*, 2008; Muller *et al.*, 2003). Since male and female chicks in our study species have similar levels of testosterone (Gil *et al.*, 2008; Müller *et al.*, 2007), the higher immune susceptibility of the former should arise by differences in hormone metabolism or function (Duffy *et al.*, 2000; Fargallo *et al.*, 2002; Møller *et al.*, 1998; Moreno *et al.*, 2001).

Most experimental injections of yolk androgens typically use one single dose, and the amount that is injected varies widely between studies (Muriel *et al.*, 2015a), thus making it difficult to identify general patterns. Very few avian studies have examined whether the immunological consequences of yolk androgens vary depending on the injected concentration. In contrast to low doses of testosterone, high doses have inhibitory effects on the growth of the bursa of Fabricius in em-

bryos (Norton and Wira, 1977), which is a central humoral immune organ that plays important roles in avian B cell development and antibody production (Davison *et al.*, 2008). Other studies have also found that high testosterone doses resulted in detrimental effects on cell-mediated immunity in nestlings (Navara *et al.*, 2006).

Theory predicts that benefits derived from high androgen levels are counteracted by negative effects in immunity (Folstad and Karter, 1992). In the case of yolk androgen levels, several studies have found support for this hypothesis, both in the cellular and humoral axes of immunity (Groothuis *et al.*, 2008; Müller *et al.*, 2005; Sandell *et al.*, 2009). However, other studies have found no effects (Rubolini *et al.*, 2006b) or conflicting evidence. For instance, in another experiment in the same species, we found that differences in lymphocyte proliferation between treatment and control nestling varied depending on the time of the season when the study was done (Muriel *et al.*, 2015b). Thus, cellular immune response actually increased in androgen-treated nestlings in first broods and showed an antagonistic trend in second broods. This suggests that environmental conditions, likely food availability, exposure to parasites or differences in egg composition profoundly affect the cost/benefit balance of yolk androgens (Muriel *et al.*, 2015b). Since differences in environmental conditions during early development may differentially affect male and female offspring (Blondel *et al.*, 2002), it is possible that the adverse weather conditions experienced during first and replacement broods in 2010, cooler and drier than what is usually experienced during this period (Muriel *et al.*, 2015b), entailed higher immunological costs, especially in androgen-treated males.

Although we found no evidence of any direct correlation between cell-mediated immune response and lysozyme activity in plasma (but see: Clairardin *et al.*, 2011), both measurements showed the same sex-specific responses to high yolk androgen levels. These results can be interpreted on the light of common components in the innate and adaptive axes of the immune system (Forsman *et al.*, 2010; Vinkler *et al.*, 2010). However, contrary to lysozyme activity, cell-mediated immune response was also influenced by several variables. Thus, we found that cell-mediated immune response was positively correlated with nestling growth; which is consistent with previous studies finding a less developed cell-mediated immune response in nestlings with lower growth rate (Horak *et al.*, 2000; Hõrak *et al.*, 1999). Since the activation of an immune response has energetic and/or nutrient costs that may interfere with metabolic processes (Demas *et al.*, 1997), the positive relationship between cell-mediated response and the increase in mass that we found suggests that birds did not trade-off one against the other.

Interestingly, although yolk androgen treatment entailed a greater immunosuppression in males, the overall cell-mediated response was greater in males than in females (López-Rull *et al.*, 2011), which might be related to differences in competitive advantage over food under harsh environmental conditions (Alonso-Alvarez and Tella, 2001). In fact, several studies have shown that variations in cell-mediated immune response may be associated with different sex-sensitivity to environmental conditions (Chin *et al.*, 2005; Dubiec *et al.*, 2006; Fargallo *et al.*, 2002).

We found that cell-mediated immune response as well as several haematological parameters, such as basophils and monocytes, decreased as the breeding season advanced, as is often the case in other studies (López-Rull *et al.*, 2011; Sorci *et al.*, 1997). We have previously shown a negative effect of clutch laying date on nestling body condition in the same study population (Muriel *et al.*,

2015a). This pattern is in agreement with the lower immune responses that we detected in this study. This pattern could arise because of lower parental quality, since high-quality birds breed earlier in the season and are probably less likely to lose their first laid clutches (Brinkhof *et al.*, 1999; Verhulst and Nilsson, 2008). In addition, differences can arise because of differences in overall food quantity and quality or differential exposure to parasites and pathogens across the breeding season (Biard *et al.*, 2015; Muriel *et al.*, 2015b; Sorci *et al.*, 1997). In agreement with this possibility, we found an increase of trypanosomes and coccidia in replacement broods compared to first broods.

In a previous study we used an in-vitro assay to study T-cell proliferation patterns in nestlings after yolk-androgen manipulation, and found that it varied depending on the season (Muriel *et al.*, 2015b). It is not possible to directly compare those patterns with the present study because here we have used a measure of the external swelling response, which not only involves proliferation of T-cells but also the secretion of proinflammatory cytokines that recruit and activate effector cells and phagocytes such as basophils, heterophils, and macrophages (Martin II *et al.*, 2006; Salaberria *et al.*, 2013), and thus involves both innate and adaptive components of the immune system (Bílková *et al.*, 2015; Stadecker *et al.*, 1977).

The different components of the immune system do not always respond in the same way and studies show that they can interact and trade-off with one another (Forsman *et al.*, 2008; Martin *et al.*, 2006; Palacios *et al.*, 2007). We found that hemolysis and agglutination (measures of natural antibody levels and complement activation, respectively: Matson *et al.*, 2005) were positively correlated with each other, as found before in other avian species (Matson *et al.*, 2006). This correlation is expected because natural antibodies, responsible for hemagglutination, are also involved in the hemolysis process, as antibodies interact with the antigen (SRBC) to create complexes on which complement proteins responsible for lysis act (Matson *et al.*, 2005). However, these two measures were negatively related to cell immunity, showing perhaps different immune strategies between individuals. It is possible that differences in environmental factors, such as food availability or weather conditions, could explain this pattern, since these two groups of immune responses showed opposite patterns in relation to breeding season (e.g. first vs. replacement clutches). Lysozime activity however behaved independently to any other component of the immune system included in this study. This is not the first time that immunocompetence estimates are found to differ, showing negative covariance. For instance, it has been found in house wrens that humoral and cell-mediated responses covary negatively among broods, suggesting a trade-off in immunity (Forsman *et al.*, 2008). In fact, a study in that same species has been shown that experimentally increased in-ovo testosterone induces a trade-off between bactericidal activity and cutaneous immune response (Clairardin *et al.*, 2011).

Thus, our results show that immunosuppression resulting from high yolk androgen levels affected components of both the innate and adaptive axes of the immune system in males. Given the complex nature of the different axes, this does not allow us to predict how other components of the immune system could be affected by the manipulation (Forsman *et al.*, 2008). Yolk androgens may prime the development and expression of various components of the immune system such as cell-mediated and humoral immune function differently (Sandell *et al.*, 2009), with sex- and context-specific consequences (Pigeon *et al.*, 2013).

Immunosuppression due to high androgen levels could make individuals more susceptible to disease. However, we

found no evidence for malaria infection in any of the individuals during the nestling phase. This could be due to blood samples being taken too early in development (14 days old), since data in other species show that the prepatent period (the time from infection to the demonstration of the parasite in the blood) is longer than the nestling period in most bird species (Cosgrove *et al.*, 2006; Fallis and Bennett, 1961; Merino and Potti, 1995). On the other hand, we found only a few chicks infected with trypanosome, species which has probably a shorter prepatent period (Merino and Potti, 1995). In contrast, the prevalence of infection by coccidia (*Isospora* spp.) was high, although there were no differences between experimental groups. In line with our results, Tschirren and co-authors found no indication that high concentrations of yolk testosterone increase the nestling's susceptibility to ectoparasites (Tschirren *et al.*, 2005). Similarly, a previous study that increased levels of androgens by subcutaneous placement of testosterone-filled implants showed that neither hormone treatment nor age influenced prevalence of coccidia (Hudman *et al.*, 2000). It is possible that the absence of effects was due to a delayed effect of androgens on coccidia infection, as it has been shown for other intestinal parasites of testosterone-implanted birds (e.g. Mougeot *et al.*, 2005). Broods in replacement broods had higher levels of both coccidia and trypanosomes. This pattern is likely due to differences in abiotic factors, such as temperature or humidity (Brooker *et al.*, 2006; Svobodova *et al.*, 2015) or to seasonal differences in diet (Peris, 1980) which would imply different exposure to parasites (Dolnik *et al.*, 2010).

To sum up, our study showed that experimentally increased yolk androgens led to sex-specific immunosuppression of two measures of innate and adaptive axes of the immune system. Despite this, there was no evidence of increased susceptibility to blood or intestinal parasites. We found a negative covariance among different branches of the immune system, suggesting that future studies about the effects of maternal androgens on immune response should consider a wider range of immunological assays which cover as many branches of the innate and adaptive immune system as possible. In addition, we note the importance of exploring the sex- and context-specific effects of yolk androgens to understand more precisely the role of maternal effects in the offspring immune response.

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Foraging in a dry land

Fruits and seeds compose an important part of the diet of the spotless starling. However the main component during spring and summer are invertebrates. Food availability decreases as the breeding season advances, and during late broods they face a harsher environmental context.

This chapter is based upon the manuscript: **Muriel, J.**, Salmón, P., Núñez-Buiza, Á, de Salas, F., Pérez-Rodríguez, L., Puerta, M., Gil, D. (2015) Context-dependent effects of yolk androgens on nestling growth and immune function in a multi-brooded passerine. *The Journal of Evolutionary Biology*, **28**, 1476-1488.

Context-dependent effects of yolk androgens on nestling growth and immune function in a multi-brooded passerine

Abstract: Female birds may adjust their offspring phenotype to the specific requirements of the environment by differential allocation of physiologically active substances into yolks, such as androgens. Yolk androgens have been shown to boost embryonic development, growth rate and competitive ability of nestlings, but they can also entail immunological costs. The balance between costs and benefits of androgen allocation is expected to depend on nestling environment. We tested this hypothesis in a multi-brooded passerine, the spotless starling, *Sturnus unicolor*. We experimentally manipulated yolk androgen levels using a between-brood design, and evaluated its effects on nestling development, survival and immune function. Both in first and replacement broods, the embryonic development period was shorter for androgen-treated chicks than controls, but there were no differences in second broods. In replacement broods, androgen-treated chicks were heavier and larger than those hatched from control eggs, but this effect was not observed in the other breeding attempts. Androgen exposure reduced survival with respect to controls only in second broods. Regarding immune function, we detected non-significant trends for androgen treatment to activate two important components of innate and adaptive immunity (IL-6 and Ig-A levels, respectively). Similarly, androgen-treated chicks showed greater lymphocyte proliferation than controls in the first brood and an opposite trend in the second brood. Our results indicate that yolk androgen effects on nestling development and immunity depend on the environmental conditions of each breeding attempt. Variation in maternal androgen allocation to eggs could be explained as the result of context-dependent optimal strategies to maximize offspring fitness.

Keywords: Yolk androgens, testosterone, androstenedione, maternal effects, *Sturnus unicolor*, immune response, life history trade-offs, breeding conditions

Introduction

Female birds deposit variable amounts of physiologically active substances into egg yolks (Ricklefs, 1984; Williams, 1994; Bernardo, 1996), which potentially affect embryonic growth and development and can vary seasonally (Hargitai *et al.*, 2009). This flexible maternal mechanism may allow females to adjust the offspring phenotype to specific requirements of the environment (Mousseau and Fox, 1998; Vergauwen *et al.*, 2012; Giordano *et al.*, 2014). Since the publication of the first study confirming the presence of maternally derived hormones in the yolk (Schwabl, 1993), elucidating the role of yolk androgens as modulators of maternal effects has been a subject of intensive research during the last twenty years (Gil

Appendix S2. Supplementary Methods.

Blood cell isolation and immune tests

White blood cell isolation. Blood was centrifuged at 3000 rpm and 4ºC for 5 minutes, just enough to allow partial plasma recovery without forming a compact pellet. Plasma was stored at -20ºC until IL-6 analysis and cells were immediately processed for WBC isolation. The isolation procedure was based in procedures already described (Strain and Matsumoto, 1991; Finkelstein *et al.*, 2003; Gil and Culver, 2011). Briefly, blood was diluted (1:1) in Roswell Park Memorial Institute 1640 medium with hepes (RPMI, Sigma, St. Louis, MO) containing 1% bovine serum albumin (BSA, Sigma, St. Louis, MO), and penicillin-streptomycin −neomycin (200 U – 0.2 mg - 0.4 mg/ml, respectively, Sigma, St. Louis, MO) and mixed gently (this mixture will be referred to as RPMI+). This mixture was set above an equal volume of a double layer of Histopaque gradient (Sigma, St. Louis, MO): HP 1.119:HP 1.077, and centrifuged at 700g during 30 minutes. The layer above HP 1.077 containing the lymphocytes was collected and transferred to a clean tube with 400 µl RPMI+ and centrifuged at 250g for 12 min. The supernatant was aspirated and the cells resuspended in 400 µl RPMI+, and thoroughly mixed to avoid cell aggregates. The final pellet was gently resuspended in 200 µl RPMI+. We counted the number of alive lymphocytes in a 15 µl aliquot mixed with 5 µl tripan blue in a counting chamber. We used this figure to finally dilute the homogenate to a final concentration of 105 lymphocytes per 100 µl .

Lymphocyte T proliferation. Lymphocyte T proliferation was measured in flat-bottom 96-well plates (Nunc, Roskilde, Denmark). Two duplicates per sample of the 200 µl of lymphocyte suspension (containing 105 lymphocytes per 100µl) were incubated with 20 µl of AlamarBlue® (AbD Serotec). This dye indicates the oxidation-reduction state of the medium, measuring both the intensity and velocity of the proliferation process. One of the two duplicates (experimental) received 20 µl of a PBS solution containing 50 µg of phytohemaglutinin (PHA) (Sigma, L8902), and the other well (control) received a similar volume of PBS. Plates were incubated at 38ºC during 72 hours, and we read absorbances at 0, 24, 48 and 72h of the process. Readings were done in a plate reader at both 570 and 600 nm and calculations performed following the instructions from the commercial kit after adjusting to our species.

To adjust the AlamarBlue procedure to our species, calculations were done considering the molar extinction coefficients of the reduced and oxidized form of AlamarBlue at the two wavelengths recommended in the in the commercial kit insert. Lymphocyte proliferation was calculated in two ways, as proliferation per se, this is, the reduction obtained once the reduction of the respective negative controls had been discounted, and as velocity of proliferation, this is, the percentage of reduction of the experimental wells compared with the respective positive controls (see commercial insert for formulae). Since results were very similar between these two types of measurement, we chose to use the more commonly used proliferation estimate. The above conditions were decided after assaying different plating densities (ranging from 10000 to 1000 cells per 100 µl) at different incubation times (24, 48 and 72h) with spotless starling blood. Proliferation increased with incubation time (Fig. S1), although between 48 and 72 hours the increase was smaller, with some cells starting to decline at 72, and thus we chose to use 48 hours as our standard point (correlation between 48 and 72 hour scores: $r = 0.930$, $N = 96$, $P < 0.001$).

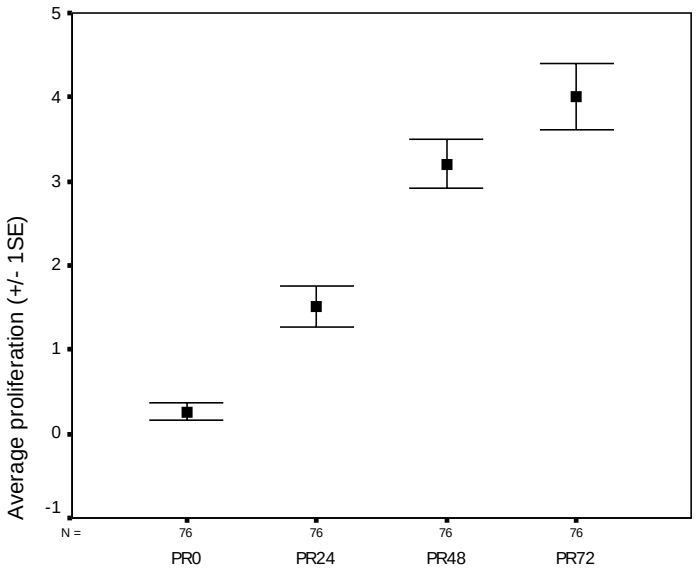

Fig. S1. Mean (±1 SE) lymphocyte proliferation as measured by the AlamarBlue technique at different time intervals since incubation: 0, 24, 48 and 72 hours.

Plasma IL-6 concentration and validation procedure. We developed an indirect ELISA using chicken IL-6 as antigen (Mybiosource, MBS 232222), a rabbit IgG anti-chicken IL-6 as primary antibody (MybioSource, MBS 220073) and goat IgG anti-rabbit IgG conjugated with horseradish peroxidase as secondary antibody (MybioSource, MBS 235191). Wells in a 96-well plate were covered with 100 µl of either standard solutions or plasma samples and left 2 hours at room temperature. Wells contents were aspirated and washed 5 times with 200 µl of Tris-buffer-saline containing 0.05% Tween 20, (TBST) pH 8.O (Sigma C3041). Inespecific binding sites were blocked with 200 µl ELISA SYMBLOCK (AbD Serotec BUF034A) for 1 h at room temperature. Wells contents were then aspirated and washed 5 times with 200 µl of TBST. Both standards and samples received 100 µl of a 5 µg/ml solution of primary antibody, left overnight at 4ºC and washed 5 times with 200 µl of TBST. Identical procedure was followed for the secondary antibody which was diluted 1:10000 in TBST with 1% albumin. After washing, 100 µl de TMB (Sigma Aldrich, Sigma T0440), were added to every well, and the plate was then incubated in darkness for 15-30 min and the reaction stopped by adding 100 µl 2M H2SO4 to every well. Absorbance at 450 nm was read in the next 30 min in a plate reader. Eight solutions containing 0.125-0.250-0.5-1-2-4-6-8 in HISPEC assay diluent (AbD Serotec BUF049A) were used as standard solutions. These conditions were chosen after tritiation experiments using 0.125-10 µg/ml as antigen solutions, 1-5 µg/ml as primary antibody solutions and 1.10000-1:50000 solution of the secondary antibody. The intra-assay variation coefficient was 6.79% and the inter-assay was 11.56%.

Fecal sampling and immunological test

IgA extraction from feces. Chicks usually defecate when manipulated for morphometric measurements and blood collection. We collected the whole fecal pellet in cold containers that were transported to the lab in 2-4 hours after delivery and conserved at -20ºC until analysis. After been taken from the freezer, they were cleaned with filter paper (the main part of the pellet of uric acid was removed) and exposed to 30-35ºC

until weight was constant. The following steps used for extraction of fecal immunoglobulin were adapted from that used by Peters *et al.* 2004. Samples were weighed and grounded with a mortar, and TBST (Tween buffer saline with 0.5% Tween 20) pH 7.4 was added in proportion 0.5 g feces/2ml TBST. They were kept under agitation for 60 min and centrifuged at 1600 g during 15 min at 4ºC. The supernatant was transferred to a sterile Eppendorf tube and mixed with a protease inhibitor cocktail (Sigma-Aldrich), in proportion 2ml extract to 20 µl cocktail and centrifuged at 10000 × g for 10 min at 4ºC for optimal removal of solid material. The supernatant was preserved at -20ºC until analysis.

IgA quantification. An ELISA quantification set developed for chicken IgA by Bethyl Lab (E30-103) was used to measure IgA in feces extracts. We followed the commercial procedure. In brief, 96-well immunoplates (Maxisorb; Nunc, Roskilde, Denmark) were covered with the first antibody, incubated at room temperature during 1 hour and washed with TBST (Tween buffer saline with 0.05% Tween 20 pH 8.0, Sigma T9039). TBS with 1% BSA (Bovine serum albumin) was used as blocking solution for a further 30 min period and washed again. A known amount of antigen (recombinant chicken IgA) or samples were added and incubated for 1 hour, the wells being washed again. The second antibody which was conjugated with HRP (horseradish peroxidase) was added and incubated for 1 hour. After washing, TMB (Sigma T0440) was added, maintained in darkness for 15 min, and the reaction stopped with H2SO4 0.18 M. The absorbance was read in a plate reader at 450nm, immediately. Both standards and samples were run in duplicate. Washing procedures were repeated 5 times throughout.

Standard solutions for the calibration curve were obtained from an initial antigen solution in TBST of 1000 ng/ml. After trying a long dilution series, we chose the central lineal part of the curve, between 400 and 6.25 ng/ml. A parallelism test was run to asses that the chicken antibodies discriminate different concentrations of IgA from Sturnus unicolor. To that end, we extracted the IgA from a pool of several fecal extract with magnetic beads so that a solution with an absorbance 2400 was obtained. Several dilutions allowed as to run a curve that paralleled to the standard curve run at the same time. R2 for different standard curves was > 98.7. The coefficients intra and inter-assay were 3.69% and 0.46%, respectively.

IgA extraction with magnetic beads for parallelism curve. Dynabeads M-270 Epoxy from Life Technologies were used to extract IgA from a pool of fecal extract. The commercial procedure was followed with minor modifications. In short, 2 x 108 beads were covered with 80 µg of the primary chicken antibody used in the previous ELISA procedure. After adding 20 µl of (NH4)2SO4 5M they were incubated for 20 hours with a vortex so that beads were not allowed to settle down. Beads were recovered under a magnetic field (Dynamag, Life technologies) washed and mix with 8 ml pool of fecal extracts. After 4 hours under agitation with vortex, they were allowed to sediment with the magnetic field, washed with PBS and resuspended in 200 ml TBST pH 8. IgA were released to the medium from the beads by adding 300 µl of citric acid 3.1 M (three sequential additions of 100 µl). Beads were settled down with the magnetic field and the supernatant was transferred to a clean eppendorf, immediately neutralized with NaOH to pH 7.4 and this solution was used to raise serial dilutions with TBST pH 8.

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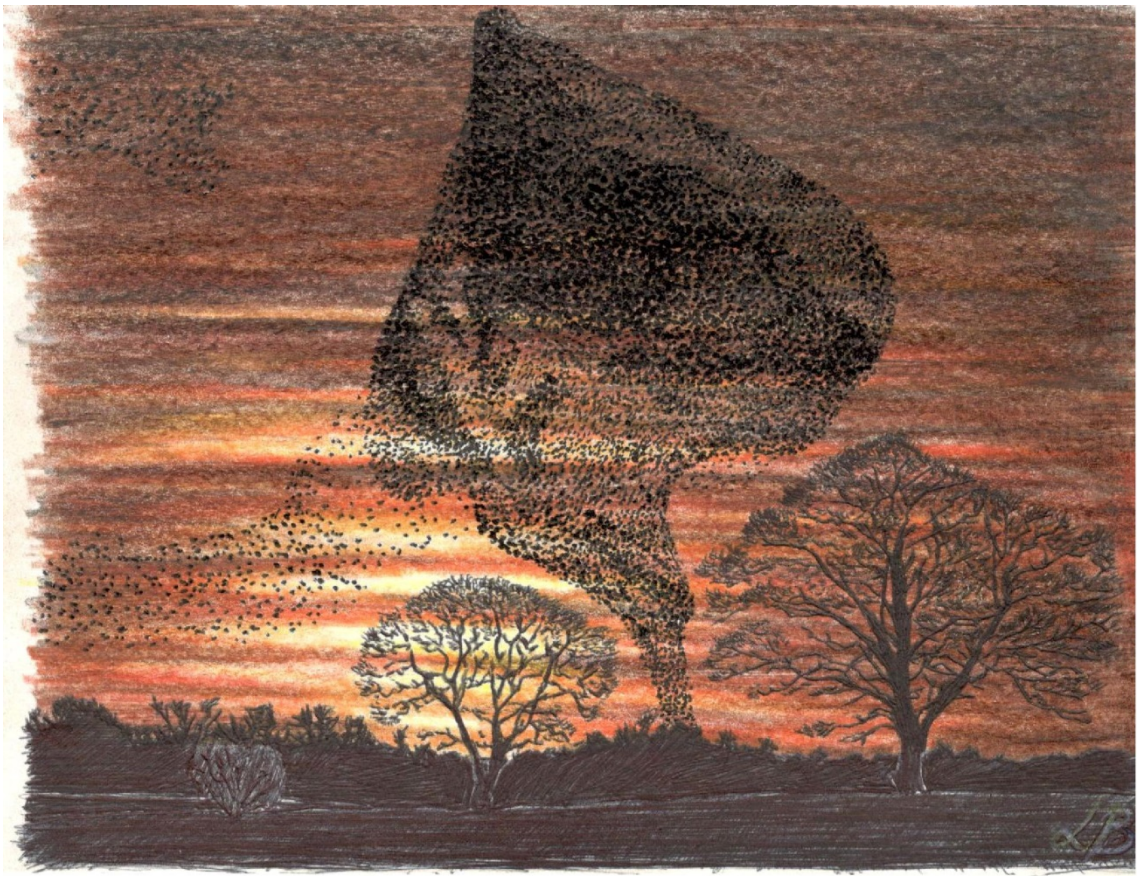
Supplementary Table S6. Fisher's LSD post hoc test of androgen treatment effects on EDP and nestling development (tarsus length and body condition) on day 14 posthatch across breeding attempts (summary statistics of final models in Table 8). Fixed factors are coded as Treat (Treatment; control: 0, androgen treated: 1) and Attempt (Breeding Attempt; first brood: 1st, replacement: R, second brood: 2nd).

Diff. between groups				EDP				Tarsus length				Body condition			
Treat	**Attempt**	**Treat**	**Attempt**	**Estimate ± SE**	**d.f.**	**t**	***P***	**Estimate ± SE**	**d.f.**	**t**	***P***	**Estimate ± SE**	**d.f.**	**t**	***P***
0	1st	0	R	-0.145±0.072	915	-2.00	0.045	0.949±0.172	428	5.51	<0.001	0.483±0.121	548	3.97	<0.001
0	1st	0	2nd	0.054±0.062	922	0.87	0.383	0.368±0.157	566	2.34	0.019	1.102±0.103	704	10.71	<0.001
0	1st	1	1st	0.115±0.053	851	2.17	0.030	0.171±0.119	386	1.44	0.151	-0.099±0.084	518	-1.18	0.240
0	1st	1	R	0.282±0.083	885	3.42	<0.001	0.133±0.189	390	0.71	0.481	0.534±0.129	503	4.11	<0.001
0	1st	1	2nd	0.059±0.039	793	1.52	0.128	0.241±0.129	929	1.87	0.062	1.263±0.078	939	16.19	<0.001
0	R	0	2nd	0.199±0.085	894	2.35	0.019	-0.581±0.202	440	-2.88	0.004	0.619±0.138	559	4.47	<0.001
0	R	1	1st	0.260±0.078	842	3.34	<0.001	-0.778±0.172	368	-4.52	<0.001	-0.582±0.124	466	-4.71	<0.001
0	R	1	R	0.427±0.100	872	4.26	<0.001	-0.816±0.227	358	-3.59	<0.001	0.051±0.160	464	0.32	0.749
0	R	1	2nd	0.204±0.076	923	2.67	0.007	-0.708±0.187	453	-3.78	<0.001	0.780±0.129	592	6.04	<0.001
0	2nd	1	1st	0.061±0.046	818	1.32	0.189	-0.196±0.145	878	-1.36	0.175	-1.201±0.088	965	-13.60	<0.001
0	2nd	1	R	0.228±0.084	931	2.70	0.007	-0.235±0.212	487	-1.11	0.269	-0.568±0.141	634	-4.01	<0.001
0	2nd	1	2nd	0.005±0.063	931	0.07	0.941	-0.127±0.171	634	-0.74	0.457	0.161±0.110	824	1.47	0.142
1	1st	1	R	0.167±0.078	917	2.15	0.032	-0.038±0.184	424	-0.21	0.836	0.633±0.126	555	5.03	<0.001
1	1st	1	2nd	-0.057±0.053	915	-1.05	0.292	0.069±0.135	600	0.51	0.608	1.362±0.089	748	15.25	<0.001
1	R	1	2nd	-0.223±0.084	907	-2.66	0.008	0.108±0.202	439	0.53	0.594	0.729±0.136	571	5.34	<0.001

Supplementary Table S7. Fisher's LSD post hoc test of androgen treatment effects on lymphocyte proliferation at 48 hours on day 14 posthatch between first and second brood (summary statistics of final models in Table 8). Fixed factors were coded as Treatment (Treat; control: 0, androgen treated: 1) and Attempt (Breeding Attempt; first brood: 1st, second brood: 2nd).

Diff. between groups				**Lymphocyte proliferation**			
Treat	**Attempt**	**Treat**	**Attempt**	**Estimate ± SE**	**d.f.**	**t**	***P***
0	**1st**	**0**	**2nd**	-0.06191±0.05353	67	-1.16	0.2516
0	**1st**	**1**	**1st**	-0.04543±0.02011	67	-2.26	0.0272
0	**1st**	**1**	**2nd**	-0.03317±0.06115	67	-0.54	0.5893
0	**2nd**	**1**	**1st**	0.01648±0.05395	67	0.31	0.7609
0	**2nd**	**1**	**2nd**	0.02874±0.02955	67	0.97	0.3341
1	**1st**	**1**	**2nd**	0.01226±0.06151	67	0.20	0.8426



The flock

The spotless starling is a gregarious species. It feeds in large flocks: this is thought to improve their feeding efficiency and to decrease predation by birds of prey. Flocks form a tight sphere-like formation in flight, frequently expanding and contracting and changing shape, seemingly without any sort of leader.

This chapter is based upon the manuscript: Long-term effects of high yolk androgen levels on fitness-related traits in a wild passerine. *Manuscript*.

Long-term effects of high yolk androgen levels on fitness-related traits in a wild passerine

Abstract: Mothers can modify the phenotype of their offspring by exposing them to different amounts of substances that influence their development. It is known that yolk androgens can alter offspring development, but the potential long-term effects of these hormones remain poorly understood, although this information is essential to fully assess the adaptive value of hormone-mediated maternal effects. In a large-scale study, we experimentally manipulated yolk androgen levels on 497 clutches of spotless starlings (*Sturnus unicolor*). In the following three breeding seasons, we monitored adult recruitment and breeding output. Also, we assessed the effects of the experimental manipulation on their plumage ornaments, morphometry and some physiological parameters (circulating testosterone and white blood cell counts). We found that yolk androgens increased the length of the throat feathers in both males and females, a key ornamental plumage trait in this species. Yolk androgens influenced survival in males: experimental males hatching late in the season showed reduced recruitment with respect to controls whereas the opposite was true for birds from early broods. This could be associated to the differential effect of yolk androgens on some components of the cellular immune response that was also detected. Despite this, among those males that succeeded to breed and produce descendants in the first year of life, the number of chicks fledged by androgen-treated males was higher than that of controls, although this effect was reverted later in life. In females, increased yolk androgens reduced clutch size, but in turn increased the egg volume. This resulted in large nestlings that also displayed wider gapes. These results demonstrate that maternally derived androgens can modify offspring adult phenotype, also affecting key life-history traits. Our study show that females can influence the outcome of many life-history trade-offs faced by their descendants, ultimately influencing individual fitness.

Keywords: Yolk androgens, testosterone, immune response, parasites, androstenedione, maternal effects, *Sturnus unicolor*.

Introduction

Beyond genes and the environment experienced by the individual, parents can modify offspring phenotype, contributing to transgenerational plasticity. Adaptive maternal effects arise when the environment or phenotype of the mother influence traits linked to offspring fitness, including morphology, physiology, behaviour, and reproductive success (Bernardo, 1996; Mousseau and Fox, 1998; Marshall and Uller, 2007, Wolf and Wade, 2009). Maternal effects can be mediated by adjustment of care or resources such as nutrients, antibodies, and hormones that are

transmitted to the developing offspring via the placenta, egg, or seed (Roach and Wulff, 1987; Schwabl, 1996; Mousseau and Fox, 1998; Grindstaff *et al.*, 2003; Maestripieri and Mateo, 2009). In this way, maternal effects can exert strong effects on ecological and evolutionary processes, improving the adaptation of offspring to future environments (Bernardo, 1996; Mousseau and Fox, 1998; Rotem *et al.*, 2003; Räsänen and Kruuk, 2007).

Oviparous species represent an ideal model to study early maternal effects due to the relative independence between mother and embryo once the egg is laid. In avian species, maternally derived androgens, mainly testosterone (T) and androstenedione (A4), have a great impact on offspring development (Schwabl, 1993). These yolk hormones play a crucial role both in the prenatal development and in a series of traits after hatching, including physiology, behaviour and life history (reviewed in Ketterson *et al.*, 1996; Clark and Galef, 1998; Weinstock, 2001; Welberg and Seckl, 2001; Dufty *et al.*, 2002; Groothuis and Schwabl, 2008). These effects can in some cases be sex-specific (Groothuis *et al.*, 2005; Gil, 2008; Ruuskanen and Laaksonen, 2010; Ruuskanen *et al.*, 2012a) and/or context-dependent (Muriel *et al.*, 2015a). Costs and benefits of yolk androgens are usually studied by experimental manipulations in which egg yolks are injected with exogenous androgens before incubation, simulating a maternal transfer in response to an hypothetical environmental stimulus (reviewed in Groothuis *et al.*, 2005; Groothuis and Schwabl, 2008; Gil, 2008; Muriel *et al.*, 2015b). The vast majority of these studies have focused on the short-term effects of yolk androgens, testing the effects of hormone manipulations in the stages between embryo and fledgling (Schwabl, 1996; Eising *et al.*, 2001; Lipar and Ketterson, 2000; Eising and Groothuis, 2003; Muriel *et al.*, 2015a, b). However, the effect of androgens on phenotypic development is not restricted to the pre- and perinatal periods; these hormones can also exert long-term effects, affecting adult phenotype and subsequent generations (Dufty *et al.*, 2002; Groothuis *et al.*, 2005; Uller *et al.*, 2007; Gil, 2008). These long-term effects of maternal hormones may also affect the rate and direction of evolutionary change (Mousseau and Fox, 1998; Räsänen and Kruuk, 2007). However, only a few studies have tested the potential persistent effects of prenatal androgen exposure into adult life (reviewed in Groothuis and Schwabl, 2008; Gil, 2008; Ruuskanen *et al.*, 2012a, b).

Trade-offs between life-history traits have a crucial effect on reproduction and survival (Stearns, 1992), and can be influenced by maternal effects. This way, the enhancing effect of androgens on growth early in life (Schwabl, 1996; Eising *et al.*, 2001; for reviews see, e.g., Groothuis and Schwabl, 2008; Gil, 2008) could boost size-dependent traits in adulthood (Müller *et al.*, 2009), such as the probability of acquiring a territory, or reproductive fitness components (reviewed in Lindström, 1999; Cam and Aubry, 2011; Müller *et al.*, 2009; Ruuskanen *et al.*, 2012a). Although it is known that androgens are intimately associated with reproduction in adulthood (Stearns, 1992; Ketterson and Nolan, 1992; Fusani, 2008), studies on long-term effect of yolk androgens fail to show consistent results. For example, there is little evidence about the long-term effects of yolk androgens on female reproduction into adulthood. Studies in which the embryo was exposed to elevated yolk androgen levels have shown both long-term effects on egg size or clutch size (Uller *et al.*, 2005; Müller *et al.*, 2009, respectively) and an absence of effects (Von Engelhardt *et al.*, 2004). If yolk androgens influence the individuals' ability to efficiently convert resources into sexual signals, then these maternally derived hormones could have a positive effect on Darwinian fitness (von Engelhardt *et al.*, 2004). On this respect, several ex-

perimental studies have reported that yolk androgens are involved in the expression of many secondary sexual characters (Strasser and Schwabl, 2004; Eising *et al.*, 2006), although a recent meta-analysis shows that the effects of yolk androgens on sexually selected male characters may be comparatively small (Müller and Eens, 2009). . On the other hand, the immunocompetence handicap hypothesis suggests that an increase in sex hormone level might negatively affect the ability to raise efficient immune responses (Folstad and Karter, 1992; Wedekind and Folstad, 1994). Yolk androgens can also induce high metabolic rates (Nilsson *et al.*, 2011; Ruuskanen *et al.*, 2013). These physiological costs in adulthood, added to those associated with increased yolk androgen levels during early life (reviewed in Gil, 2008; Navara and Mendonça, 2008), could undermine survival (Birkhead *et al.*, 2000; Sockman and Schwabl, 2000; but see Eising and Groothuis, 2003; Müller *et al.*, 2007or local recruitment (Ruuskanen *et al.*, 2012a; but see Hegyi *et al.*, 2011). In short, there is no clear direction of the effects of yolk androgens on offspring reproduction and survival, and given the paucity of studies on this line, it is essential to conduct experimental studies to place the role of maternal hormones in the context of life-history trade-offs into adulthood.

From a broader perspective, evidence shows that maternal effects could alter offspring life-history traits to maximize own maternal lifetime reproductive success, provided that their short and long-term consequences have an adaptive value (Mousseau and Fox, 1998; Wilson *et al.*, 2005). However, the mechanistic and transgenerational approaches underlying the consequent organizational effects remain largely unknown (Carere and Balthazart, 2007; Groothuis and Schwabl, 2008; Schweitzer *et al.*, 2013). As mentioned before, the adaptive effect of hormone-mediated maternal effects is more evident on early stages of life (Groothuis *et al.*, 2005; Gil, 2008; Love and Williams, 2008); however, despite recent studies that analyze their long-term fitness-related effects (see above), there are few studies addressing their adaptive value into adulthood in the wild (Groothuis and Schwabl, 2008). Most log-term analyses have been conducted in captivity, where it is difficult to assess the adaptive value of the manipulation (e.g. von Engelhardt *et al.*, 2004; Rutkowska *et al.*, 2007; Müller *et al.*, 2009; Schwabl *et al.*, 2012; Schweitzer *et al.*, 2013), and patterns may strongly differ from those found in studies in the wild. For instance, a significant association between the strength and direction of sexual size dimorphism and sex-specific sensitivities to yolk androgens has been observed in studies performed in the wild, while there was no evidence of this relationship in captivity (Tschirren, 2015). Yolk androgen consequences on survival and reproductive success are still unclear in adult stage, being difficult to discern whether they are carry-over effects of effects that are selected in early life (Groothuis and Schwabl, 2008) or, the direct target of selection of hormone-mediated maternal variation. Therefore, further long-term empirical studies, particularly performed in natural conditions, are required to uncover the real value of maternal androgens as mediators in phenotype adaptive plasticity.

Here, we used an integrative experimental approach to explore the long-term effects and the life-history trade-offs associated to yolk androgens. We manipulated yolk androgen levels of an entire wild breeding colony (464 clutches) of spotless starlings (*Sturnus unicolor*) and monitored the recruits of that cohort in the following three breeding seasons (2012, 2013 and 2014). Our main aim was to analyze the fitness consequences of the treatment over time, evaluating long-term effects on adult morphology, survival and reproduction. We also explored the effects of the experimental treatment on the adult levels of circulating androgens and the

Figure 22. When spotless starlings are in full breeding status, both male and female beaks are yellow. However, the base of the male's beak has a bluish area and the female's a pink one. *Picture credit: Ginés Toral.*

expression of a key secondary sexual trait in this species, the length of the throat feathers (as sexual ornament, Aparicio *et al.*, 2001; López-Rull *et al.*, 2007), as proxies of individual investment in sexual signalling. Finally, we also tested the effects of yolk androgen manipulation on some haematological indices of physiological stress and immune function at adulthood.

Material and Methods

Study area and study species

The study was conducted during three consecutive breeding seasons (from 2012 to 2014) in a nest-box colony of spotless starlings located in a mixed oak and ash woodland in central Spain (Soto del Real, Madrid). The spotless starling is a medium-sized facultative polygynous and hole-nesting passerine (Moreno *et al.*, 1999; Veiga, 2002), which shows high breeding synchrony. It is a moderately long-lived species with maximum lifespans of 8 or 9 years (Veiga and Polo, 2011). Regarding sexual dimorphism, adult males are 6% heavier and have wings 3% longer than adult females (Cordero *et al.*, 2001; Fig. 22). In addition, throat-feather length is 41% longer in males than in females, and is a male secondary sexual trait that predicts male attractiveness and reproductive success (Aparicio *et al.*, 2001; López-Rull *et al.*, 2007; Fig. 23). In our study population, the spotless starling is commonly double brooded, laying the first clutch around mid-April and the second clutch towards the end of May (Salaberria *et al.*, 2014; Muriel *et al.*, 2015a). If loss of the first clutch occurs due to sabotage by conspecifics, females usually lay a replacement clutch (Müller *et al.*, 2007; Muriel *et al.*, 2015a). As a general matter in our study area, breeding conditions become harsher as the season advances, leading to higher temperatures and scarce food (i.e. from first to second broods, see Muriel *et al.*, 2015a). Modal clutch size is 5 eggs (López-Rull *et al.*, 2007), and fledglings leave the nest around 22 d of age (Cramp, 1998). Both sexes share chick feeding and nest cleaning, but male contribution can vary from 50% to 0%, being particularly low in the case of secondary nests of polygynous males (Jimeno *et al.*, 2014; Veiga *et al.*, 2002).

Yolk androgen-manipulation and nestling measurements

Yolk hormone manipulations were conducted in 2011 and survivors were monitored during the breeding seasons 2012, 2013 and 2014. In short, all clutches laid during the 2011 breeding season were randomly injected either with control or androgen treatment as described elsewhere (Muriel *et al.*, 2015a). In the andro-

Figure 23. During courtship, male starlings conspicuously display throat feathers while singing from exposed perches. **Inset:** Sexual dimorphism in throat feathers. *Picture credits: Stephan Peten.* Inset: *Lara Moreno.*

gen clutches (N= 246), eggs were injected with a mixture of yolk androgens corresponding to 4 standard deviations of the natural average found in the same population (testosterone: 14 ng per yolk [SD = 6.0], androstenedione: 50 ng per yolk [SD = 17.1]; D. Gil, unpublished), adjusted for mean yolk mass (average yolk mass 1.4 g). The mixture of hormones was composed of 24 ng T (ref. 86500, Sigma-Aldrich, Steinheim, Germany) and 68 ng A4 (ref. A9630, Sigma Aldrich), dissolved in 10 µl of sesame oil (ref. 85067, Sigma-Aldrich). In control clutches (N= 251), eggs were injected with 10 µl of sesame oil alone. These androgen levels were selected based on results of a previous dose-dependent study carried out in the same experimental population, where the chosen dose entailed a stimulatory effect on hatching body mass and nestling skeletal growth (Muriel *et al.*, 2015b). In ovo injections were performed in the field using standard U-50 insulin syringes (Terumo Corporation, Tokyo, Japan) following a standard protocol (for more details on injection protocol see Muriel *et al.*, 2015a).

Ten days after the beginning of incubation, nests were visited daily to check hatching time and hatching success. After determining the exact hatching date, all nestling biometric measurements were taken on day 14 post-hatching, when all chicks were ringed with numbered aluminum bands, and a uniquely identified micro-transponder was placed under the skin of their backs (Trovan ID-100A; Dia. 2.12 mm; Length 11.5 mm; mas 0.09 g. Trovan Ltd., Douglas, UK). These microchips helped us to detect in subsequent years surviving individuals that we were not able to capture in the prelaying period (see

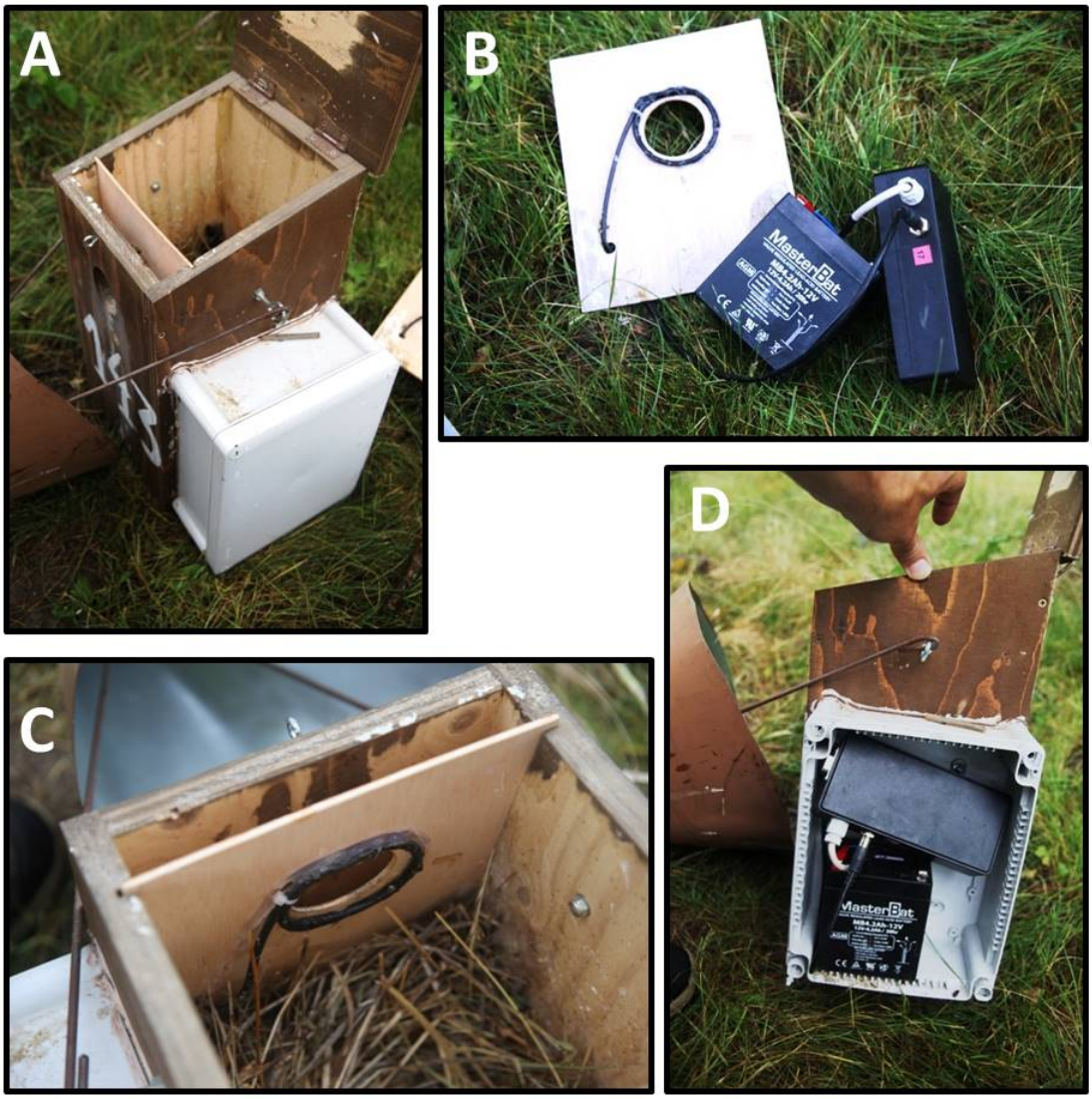


Figure 24. Each nest-box is equipped with a lateral watertight compartment (A) which contains a battery and a data-logger connected to an antenna (B). The antenna is attached to the nest-box entrance and records visits by birds that carry transponders (C). Detail of reader and battery placement inside the watertight compartment (D). *Picture credit: Diego Gil.*

below). Out of all injected eggs, the final sample sizes were 458 control and 519 androgen-treated fledglings.

Survival monitoring and measurements

Our spotless starling colony comprises a total of 250 nest-boxes. In the following three breeding seasons (2012-2014), from early March to early July, we recorded entrances to the nest box by birds with microchips with the aid of 30 data-loggers equipped with an antenna (Trovan, EID Iberica, Madrid, Spain; Fig. 24). Data gathered allowed us to detect survivors and to identify the parents of each clutch or brood. Sampling effort was similar in the different years: before laying, each box was sampled for at least two mornings; and during chick feeding for at least one morning. In addition, we used hand-held data-loggers to identify all incubating females at night. During the pre-laying period (from early May until the first egg of the colony was laid, usually in early

April) male and female starlings were caught by traps placed inside nest-boxes. From every individual captured, a blood sample for hormone assays was taken from the jugular vein (approximately 0.7 ml) with heparinized syringes immediately when extracted from the trap. Blood samples were kept cold (approx. 4°C) until the adequate processing in the laboratory within 6 hours, where plasma and red cell fractions were separated by centrifugation (at 10,000 r.p.m. for 10 min) and stored at -80 °C until analysis. A drop of blood was smeared on individually marked microscope slides and air-dried.

We recorded body mass with a digital balance (Ohaus Scout II SC2020, China, accuracy = 0.1 g), tarsus length with a digital calliper (Mitutoyo Absolute, Japan, accuracy = 0.01 mm) and wing length with an end-stop ruler (accuracy = 1 mm). Three feathers were carefully plucked from the throat, aiming at selecting those that were the longest. Feathers were kept in plastic bags until length measurements were performed with digital callipers in the laboratory. The lengths of the three feathers collected from each individual were consistent, as reflected by the high repeatability obtained for both sexes (males: r = 0.90, females: r = 0.87). For the analyses, we used the average length of these three feathers, which also showed a very high repeatability between-samples in the same year (males: r = 0.995, females: r = 0.966; n = 32 for each sex).

For the analysis of survival, we pooled data from all captured individuals and those detected by data-loggers and never caught. We detected a total of 259 experimental survivors in total in the three study years (243 survivors in 2012, 8 new survivors in 2013 and 5 additional ones in 2014). Breeding data was similarly collected in the three years of the study: nest-boxes where at least an experimental recruit was identified (male of female) were monitored by the same person (JM), who recorded: laying date, clutch size, time to hatch, brood size, fledgling success, and nestling body mass and tarsus length at 14 days post-hatching. Fledgling success was recorded as the difference between initial clutch size and the number of dead chicks found in the nest up to 25 days old. Because of the context-dependent effect of yolk androgens reported in a previous study (Muriel *et al.*, 2015a); we took into account both the breeding event in which survivors were hatched in 2011 (early and late broods), as well as the breeding events in which they reproduced in the following breeding seasons (first, replacement and second broods). Due to clutch sabotage or brood mortality, not all individuals that attempted breeding and produced a clutch finally succeeded to produce fledglings. Breeding success was computed as the sum of fledglings per year for those nests with at least one fledgling.

Testosterone assays and leukocyte counts

Testosterone was extracted from 0.12 ml plasma samples with 2 ml diethyl ether. After shaking tubes on a multi-tube vortexer for 15 min and a subsequent centrifugation for 10 min (at 4°C and 1,500 r.p.m.), the bottom of the tube was immersed on a bath of ethanol and dry ice to freeze the plasma, so that the ether phase was decanted out and dried in a bath of 37 °C. This procedure was done twice. The overall extract was resuspended in 0.2 ml of assay buffer (Cayman Chemicals, Ann Arbor, MI). Hormone concentrations were determined in duplicate using a commercially available enzyme immunoassay (Cayman Chemicals) following the manufacturer's protocol. The assay is 100% specific for testosterone, 27.4% for 5a-dihydro-testosterone (5a-DHT), and 3.7% for androstenedione. However, as 5α-DHT and T have similar receptor affinities, we consider that our measure of T is not biased by the eventual presence of 5α-DHT in the samples. For each breeding season, internal standards were also run in

each plate to adjust for inter-assay differences. Inter- and intra-assay coefficients of variations were, respectively, for 2012: 6.52% and 7.87%, 2013: 11.82% and 11.34%, and 2014: 10.51% and 8.20%. We analysed the linearity of our plasma samples by carrying out serial pool dilutions, which provided a slope that did not differ from the expected value. Hormone levels were no normally distributed and were therefore log transformed.

We used blood smears to estimate number of total and differential WBC counts (Walberg, 2001). Blood smears collected in the field were fixed with absolute methanol and stained with commercial Giemsa diluted with PBS pH 6.8 (1:2) for 45 min. Slides examined under microscope (1,000x magnification with oil immersion) to estimate the proportion of different types of leukocytes (Campbell and Ellis, 2007). Examination continued until 100-120 leukocytes had been found per slide (Salaberria *et al.*, 2013). Differential white blood cell (WBC) counts provides an estimate of innate immune response characteristic for cell-mediated processes in response to infections (Norris and Evans, 2000) that is easily obtained without involving prolonged manipulation of wild animals. We also calculated the heterophil:lymphocyte (H/L) ratio, an index that is positively associated with a levels of physiological stress in birds (Gross and Siegel, 1983; Maxwell and Robertson, 1998).

Statistical analyses

All statistical analyses were conducted with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). A new variable to summarize the structural size of individuals was derived from the first component of a Principal Components Analysis carried out on wing and tarsus lengths (Rising and Somers, 1989). Given the sexual dimorphism of this species, we performed this analysis separately by sex and year. The first component of this analysis ("body size" hereafter) had positive loadings for both source variables, explaining between 57.85 and 84.41% of their variance. Biometric variables (such as body size, body weight and length of throat feathers) were analyzed using mixed models for repeated measures (SAS, Proc Mixed, normal distribution), with Satterthwaite correction to adjust the degrees of freedom. In order to control for non-independence of individuals from the same brood, nest of origin was defined as a random effect affecting the model intercept, and the identity of the individual was entered as repeated factor. The following variables were included as fixed factors in the main model: treatment, sex, age, breeding event (early, replacement, or late clutches), capture date and body size (except when it was the dependent variable). Given that body size was included as a covariate in the analyses of body weight, the results of these models were interpreted as "body condition" (Garcia-Berthou, 2001). Treatment (Control vs T+A4), Sex (male vs female) and breeding event (early and late broods) were considered as categorical variables. All biologically meaningful double and triple interactions were also included in initial models. Specifically, since we expected treatment effect to change along age and differ between sexes, the triple interaction treatment×sex×age was included in all initial models. Statistical parameters of the final models and their estimates (± SE) are shown in the tables 9 and S8, respectively. When required, we explored the differences between categorical interactions by using Fisher's LSD post-hoc tests.

The effect of yolk androgen manipulation on the probability of survival in the following years (2012-2014) was tested using generalized linear mixed models for repeated measures (Proc GLIMMIX, binomial distribution and logit link). Statistical analysis did not require correction for imperfect detection because all survivors originated in the same cohort and 95% of them were detected in their first

year of life. Due to the different reproductive biology between sexes (see Results), we tested the effect of androgen treatment and its interaction with age on survival for males and females separately. The potential effect of body size at fledging and the breeding event where birds hatched where always tested as explanatory variables. Using the same statistical approach described above, we analyzed the effect of treatment on breeding attempts of recruits, and whether those attempts were successful or not at achieving at least one fledgling (breeding success). The nest of origin of the recruits (2011) was also included as a random intercept. In addition, in order to test whether recruit biometry (weight, body size, condition and the length of the throat feathers) could mediate the potential effect of the treatments on breeding attempts and success, we repeated this last analysis with those individuals that were captured each year.

We followed the same model construction scheme detailed above to analyse breeding parameters (clutch size, egg volume and fledging success) and biometric parameters of broods raised by our recruits (average tarsus length, body mass and condition at 14 days old per brood) in each breeding event and year. Breeding parameters were separately analyzed for 2012 (the first breeding year of recruits) but also following a repeated measurements design including all the data from 2012 to 2014 in order to test the temporal trend as birds aged.

We analyzed the effect of yolk androgen manipulation on the length of ornamental feathers, testosterone levels and differential WBC counts from 2012 to 2014 using linear mixed models including treatment, sex, age and breeding event, capture date, body size and condition per year. Due to sample size limitations, it was not possible to test age-related changes with a repeated measures design. In the case of WBC counts, analyses were restricted to 2012 and 2013, as data for 2014 were not available. Testosterone levels were log-transformed to achieve normality. Additionally, arcsine square-root and logarithmic transformations were applied to leukocyte proportions and H/L ratios, respectively.

Non-significant ($P > 0.05$) terms were sequentially removed from the initial models, starting with interactions, following a backward stepwise procedure, until only the significant explanatory variables or interactions were retained in the models. When an interaction resulted significant, each single factor included in the interaction was also retained in the final model. In all cases, we assessed the validity of final models by exploring the distribution of residuals. When it was necessary to assess the biological significance of the differences tested, we also calculated effect sizes separately for each sex.

Results

Adult biometry

We found the expected sexual dimorphism in body size and weight with males being the larger sex, although both variables were also positively affected by age (Table 9, for estimate ± SE see supplementary Table S8). There was no effect of the androgen-manipulation on body size or weight ($F_{1,129} = 0.21$, $P = 0.646$, and $F_{1,118} = 0.79$, $P = 0.377$; respectively). Body weight and body condition were positively influenced by capture date (Tables 9 and S8). Finally, body condition did not differ either between treatments ($F_{1,112} = 1.76$, $P = 0.187$), sexes ($F_{1,142} = 1.18$, $P = 0.280$) or ages ($F_{1,176} = 2.85$, $P = 0.093$).

The length of ornamental throat feathers was affected by our yolk androgen manipulation: experimental birds had longer throat feathers than controls (Tables 9 and S8). Males had longer feathers than females (Tables 9 and S8; Fig. 25), but the interaction with treatment was not retained in the model, indicating that both males and females were similarly affect

Table 9. Summary of final repeated-measures mixed models showing the effect of yolk androgen treatment on adult biometry (body size, body weight, body condition and ornamental feather length). Models were run using Proc Mixed (SAS) with Satterthwait correction to adjust the degrees of freedom.

	Body size			Body weight			Body condition			Ornamental feather length		
Independent variable	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***
Treatment	–	–	–	–	–	–	–	–	–	1/122	7.08	0.008
Sex	1/136	41.16	<0.001	1/148	44.68	< 0.001	–	–	–	1/147	473.39	< 0.001
Age	1/76.9	7.92	0.006	1/174	4.54	0.034	–	–	–	1/145	153.91	< 0.001
Capture date	–	–	–	1/171	10.67	0.001	1/149	7.38	0.007	–	–	–

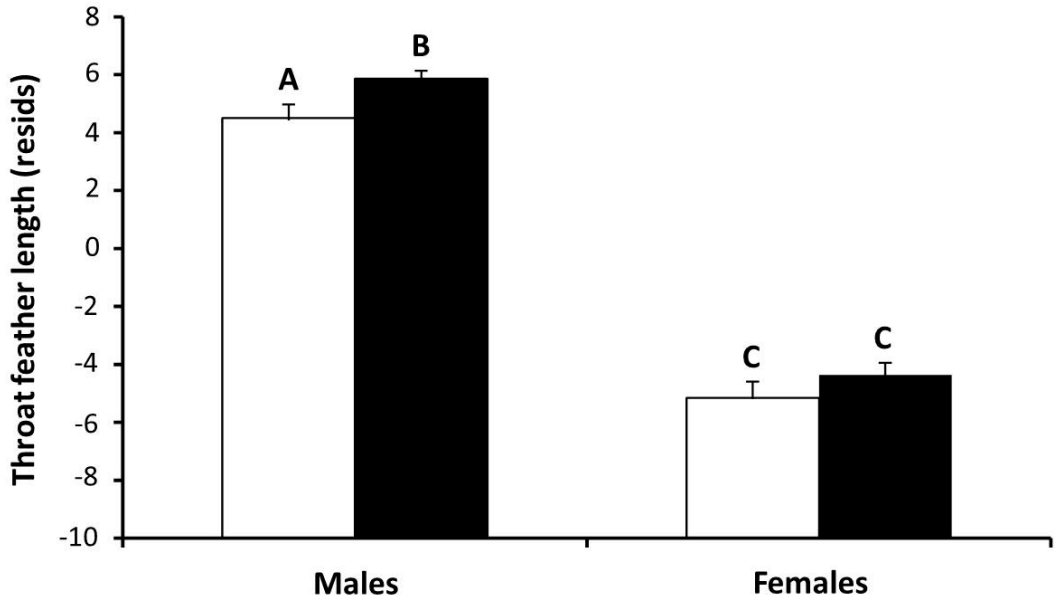


Figure 25. Differences in length of ornamental throat feathers shown as residuals from final statistical models, according to treatment and sex (white bars: control and black bars: androgen treated). Different letters above bars indicate significant differences (P < 0.05) among groups based on Fisher's post hoc comparisons.

by yolk androgen manipulation. Age was also correlated with this trait (Tables 9 and S8), indicating that throat feathers became longer as the birds aged.

Testosterone levels

Plasma testosterone concentration could be measured in 159 out of the 197 recruits caught (17 males and 45 females in 2012, 42 males and 26 females in 2013, and 20 males and 9 females in 2014). After controlling for the assay plate ($F_{11,143} = 16.04$, $P < 0.001$), male recruits showed higher plasma testosterone concentrations than females ($F_{1,127} = 122.47$, $P < 0.001$, estimate ± SE (males) = 10.422 ± 0.479). However, there was no difference in testosterone levels between recruits from

androgen-treated and control-treated eggs ($F_{1,99.6}$ = 1.22, P = 0.271). Testosterone concentration did not depend on the interaction between treatment and sex or treatment and age either (all P > 0.633).

Differential WBC counts

Unfortunately, we could only carry out leukocyte counts in 2012 (n = 47) and 2013 (n = 64), but not in 2014. Recruits hatched from androgen-treated eggs showed higher proportion of eosinophils than controls ($F_{1,61.9}$= 4.87, P = 0.031, estimate ± SE (controls) = -0.029 ± 0.0134). This leukocyte type increased with age ($F_{1,99.2}$= 19.21, P < 0.001, estimate ± SE = 0.071 ± 0.016), and was negatively affected by capture date ($F_{1,107}$= 10.16, P = 0.002, estimate ± SE = -0.002 ± 0.001). The percentage of lymphocytes was marginally affected by an interaction between treatment, age and sex ($F_{1,96.6}$ = 3.87, P = 0.052, estimate ± SE (controls, males) = -0.124 ± 0.063), where androgen-treated males showed a tendency to have a lower proportion of lymphocytes compared to controls at younger ages ($F_{1,46.8}$= 5.05, P = 0.029), but not between experimental females ($F_{1,47.5}$= 0.17, P = 0.677). Additionally, the H/L ratio was affected by a non-significant interaction between treatment and age ($F_{1,98.4}$= 3.16, P = 0.078, estimate ± SE (controls) = 0.130 ± 0.073): androgen-treated recruits showed a slightly higher H/L ratio than controls in the first year and lower ratio in the second year.

Percentages of heterophils, basophils and monocytes were similar among experimental groups (all P > 0.10), although they were all significantly higher in the first year than in the second ($F_{1,108}$ = 7.78, P = 0.006; $F_{1,66.4}$ = 119.60, P < 0.001 and $F_{1,97.5}$ = 128.92, P < 0.001, respectively). Proportion of heterophils was also affected by date of capture ($F_{1,108}$= 7.25, P = 0.008, estimate ± SE = 0.002 ± 0.001), showing higher values as the mating season progressed.

Survival

Pooling information from trapping events and identifications by transponder readings, we were able to detect 259 different experimental survivors in total. The overall survival rate in 2012 differed between sexes ($F_{1,724}$ = 4.78, P = 0.029, estimate ± SE (males) = 0.324 ± 0.148), males showing higher recruitment rates than females. Given the sexual differences in survival, we performed statistical models for males and females separately, thus reducing the complexity of the analyses. Hence, male survival rate was affected by an interaction between treatment and the breeding event from which birds were hatched in 2011 ($F_{1,537}$ = 6.23, P = 0.013; Fig. 26): males hatched from androgen treated eggs in late clutches showed lower recruitment rates than those hatched in early clutches ($F_{1,537}$ = 2.00, P = 0.046), also showing marginally lower survival rates than controls in late clutches ($F_{1,537}$ = 1.94, P = 0.052), but there were no differences between experimental and controls from early clutches ($F_{1,537}$ = -1.59, P = 0.113; Fig. 26). Female recruitment, however, was not influenced by yolk hormone treatment ($F_{1,529}$ = 0.01, P = 0.93), either alone or in interaction with breeding event ($F_{1,526}$ = 0.24, P = 0.622). In both sexes, survival decreased with age (males: $F_{1,1081}$ = 95.07, P < 0.001, estimate ± SE = -0.572 ± 0.059; females: $F_{1,1059}$ = 92.03, P < 0.001, estimate ± SE = -0.667 ± 0.069).

Reproduction

Considering surviving individuals, we observed an interaction effect between age and sex on the number of breeding attempts ($F_{1,464}$ = 5.69, P = 0.017): although both sexes increased their reproductive attempts at older ages (estimate ± SE (males) = 0.977 ± 0.410), males showed much lower probability to attempt breeding than females at their first year post hatch. Breeding attempts were also affected significantly by the breeding event in which these individuals hatched in 2011

Table 10. Summary of final repeated-measures mixed models showing the effect of yolk androgen treatment on female breeding parameters (clutch size, egg volume, and offspring -F1- tarsus length, body condition fledging success), both for the first breeding season as all breeding seasons pooled. Models were run using Proc Mixed (SAS) with Satterthwaite correction to adjust the degrees of freedom.

		Clutch size			**Egg volume**			**F1 Tarsus length**			**F1 body condition**			**Fledging success**		
	Independent variable	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***
First breeding season (2012)	Treatment	1/82	4.80	0.031	1/74	4.22	0.043	1/35	5.77	0.0217	–	–	–	–	–	–
	Breeding attempt	2/82	8.98	< 0.001	–	–	–	2/28.4	9.17	< 0.001	2/17.8	7.12	0.005	2/75.4	5.89	0.004
	Treat × Breeding attempt	–	–	–	–	–	–	2/28.4	5.28	0.011	–	–	–	–	–	–
	Brood size	–	–	–	–	–	–	–	–	–	1/36	26.89	< 0.001	–	–	–
All breeding Seasons (2012-2014)	Treatment	1/57.3	2.99	0.089	–	–	–	–	–	–	–	–	–	–	–	–
	Breeding attempt	2/204	11.75	< 0.001	–	–	–	2/140	8.21	< 0.001	2/135	15.08	< 0.001	2/214	3.99	0.019
	Age	1/221	42.79	< 0.001	–	–	–	1/148	7.64	0.006	1/141	7.86	0.006	1/220	38.09	< 0.001
	Brood size	–	–	–	–	–	–	–	–	–	1/137	14.65	< 0.001	–	–	–
	Egg volume	–	–	–	–	–	–	–	–	–	1/120	5.29	0.023	–	–	–

Figure 26. Differences in male survival rate (i.e. residuals from final statistical models) according to treatment and breeding event from which they hatched in 2011 (white bars: control; black bars: androgen treated). Different letters above bars indicate significant differences ($P < 0.05$) between treatment groups based on Fisher's post hoc comparisons.

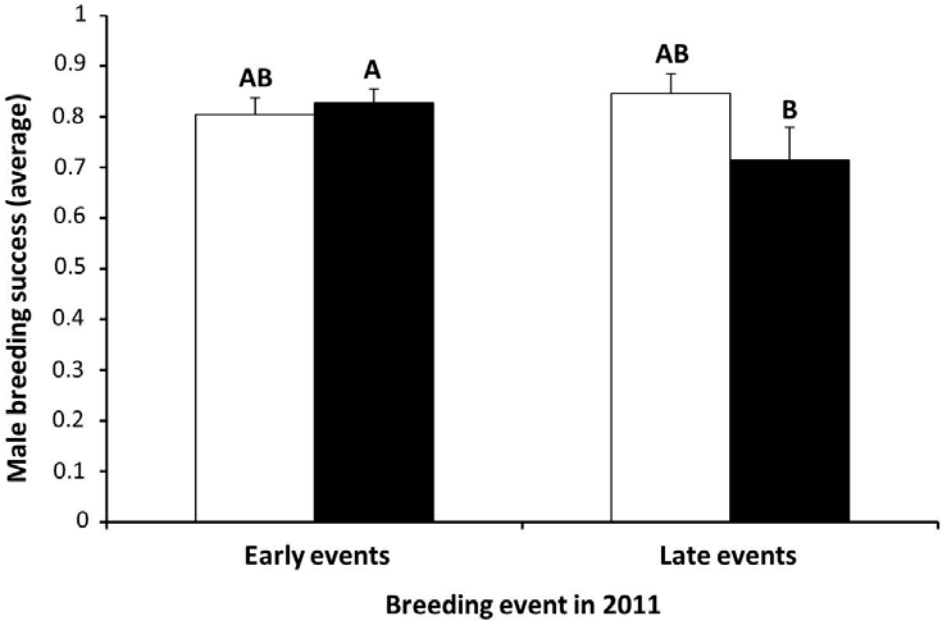


Figure 27. Differences in male breeding success shown as average per group, according to treatment and the breeding event from which they hatched in 2011 (white bars: control; black bars: androgen treated). Different letters above bars indicate significant differences ($P < 0.05$) among treatment groups based on Fisher's post hoc comparisons.

($F_{1,464} = 4.2$, $P = 0.041$): birds hatched in early clutches had a higher number of reproductive events than those hatched later (estimate ± SE (early) = 0.541 ± 0.265). When analysing both sexes separately, only males showed the above reported positive effect of age ($F_{1,263} = 75.71$, $P < 0.001$, estimate ± SE = 2.331 ±

Table 11. Summary of final repeated-measures mixed models showing the effect of yolk androgen treatment on male breeding parameters (offspring -F1- tarsus length, body condition, and fledging success) for all breeding seasons. Models were run using Proc Mixed (SAS) with Satterthwaite correction to adjust the degrees of freedom.

		F1 Tarsus length			F1 body condition			Fledging success		
	Independent variable	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***
Subsequent years (2012-2014)	Treatment	–	–	–	–	–	–	1/141	4.12	0.044
	Breeding event	2/98.6	3.51	0.033	2/79.7	22.87	< 0.001	–	–	–
	Age	1/112	8.00	0.055	1/87.3	25.07	< 0.001	1/148	15.82	< 0.001
	Treat × Age	–	–	–	–	–	–	1/148	4.12	0.044
	Brood size	–	–	–	1/74.5	3.34	0.072	–	–	–

0.268). Although less markedly than males, females also increased the number of breeding attempts per year as they aged ($F_{1,202}$ = 18.36, P < 0.001, estimate ± SE = 1.329 ± 0.310).

When analysing male breeding success, we found a significant interaction between treatment and the breeding event in which birds had hatched in 2011 ($F_{1,92}$ = 6.06, P = 0.016; Fig. 27). Androgen-treatment had a detrimental effect on breeding success in those males hatched from late clutches, ($F_{1,92}$ = 2.02, P = 0.047, estimate ± SE = 2.890 ± 1.434), whereas there was a non-significant effect in the opposite direction in males hatched from early clutches ($F_{1,92}$ = 1.88, P = 0.063, estimate ± SE = 2.079 ± 1.107; Fig. 27). In contrast, female breeding success was not affected by any of the variables considered (all P > 0.53).

Breeding parameters

Given the reproductive differences shown between males and females, we analyze the effect of our androgen treatment on breeding parameters separately for each sex and differentiating between first year and subsequent years.

In the case of male parents, no breeding parameter was affected by treatment or any other male variable in 2012 (probably due to the reduced number of males that successfully breed at their first year of life (n = 3). On the other hand, for females at first reproductive year, clutch size and egg volume were affected by the androgen treatment (Tables 10 and S9), androgen-treated females laying fewer -but larger- eggs. Clutch size also varied among breeding events (Tables 10 and S9): the number of eggs per nest in second clutches was lower than in firsts ($F_{1,82}$ = 3.00, P = 0.004, estimate ± SE = 0.745 ± 0.248) and replacement clutches $F_{1,82}$ = 4.17, P < 0.001, estimate ± SE = 1.054 ± 0.253). Regarding offspring (F1), nestlings from first and replacement clutches had longer tarsi than those hatched from second clutches (all P > 0.001). Tarsus length was affected by the interaction between treatment and breeding event in which they had hatched (Tables 10 and S9), showing that nestlings raised by androgen-treated females had longer tarsi than those of control females, but only in second broods ($F_{2,27.6}$ = 3.26, P = 0.003, estimate ± SE = -2.252 ± 0.691; Fig. 28). Fledging success and nestling condition were not affected by the androgen egg treatment administered to the mother (all P > 0.27). In general, nestlings that were raised in larger clutches were lighter ($F_{1,38}$ = 19.42, P < 0.001, estimate ± SE =

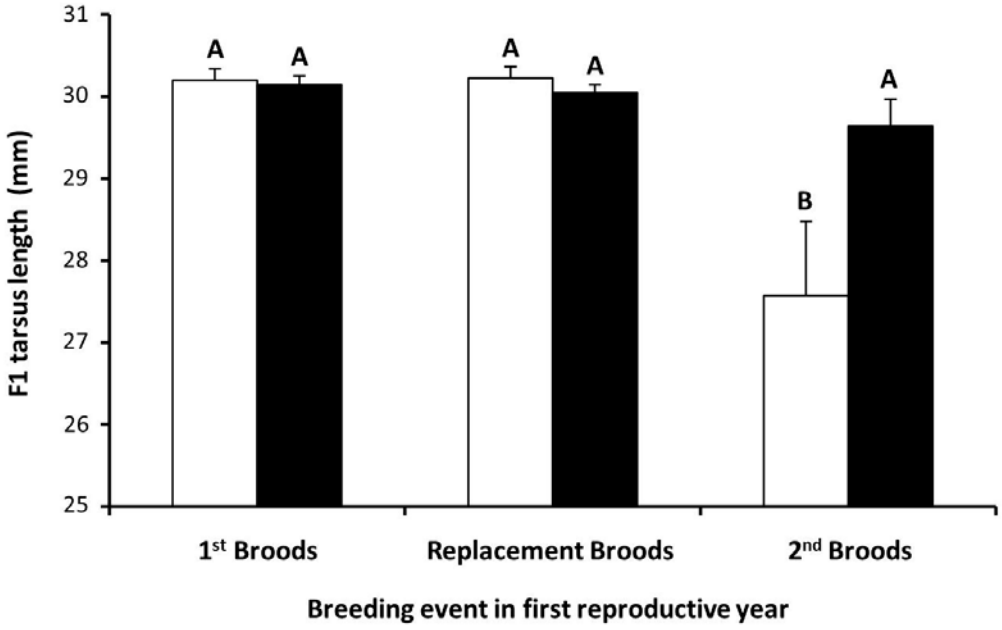


Figure 28. Average offspring (F1) tarsus length (mm), according to mother treatment and the breeding event from which nestlings hatched during the first reproductive year of females (white bars: control; black bars: androgen treated). Different letters above bars indicate significant differences ($P < 0.05$) among treatment groups based on Fisher's post hoc comparisons.

Figure 29. Differences in the average number of fledglings that left the nest, according to treatment and age of male recruits (broken line: control; solid line: androgen treated).

-4.699 ± 1.066). Also, those that hatched in second clutches had a lower weight ($F_{2,38}$ = 11.99, P < 0.001, estimate ± SE (First) = 20.190 ± 4.135, estimate ± SE (replacement) = 18.655 ± 4.252) and a worse body condition (Tables 10 and S9) than the rest. Similarly, fledging success was affected by the breeding event where chicks were raised (Tables 10 and S9), showing higher fledging success in first and replacement broods as compared to second broods ($F_{2,68.8}$= 3.03, P = 0.034, estimate ± SE = 0.291 ± 0.096 and $F_{2,81.5}$ = 3.06, P = 0.030, estimate ± SE = 0.303 ± 0.099, respectively).

In order to assess whether our treatment affected the reproductive parameters of birds in subsequent years, we performed a repeated measures analysis for the three breeding seasons (2012-2014). For male parents, fledgling success was explained by an interaction between treatment and age (Tables 11 and S10): the number of fledglings increased at older ages, androgen-treated males showing higher fledgling success in the first and second breeding year, whereas in the third year, the pattern was inversed (Fig. 29). However, nestling body size and condition were not affected by male treatment (all P > 0.74); although both biometric variables were influenced by male age and the breeding event where chicks were raised (Tables 11 and S10). As males grew older, their nestlings were larger and had better body condition. The best scores for body condition were observed in chicks from replacement broods, as opposed to those developed in second broods (replacement > first > second broods, all P < 0.008), although this pattern was slightly different for body size (replacement > first = second broods, all significant P < 0.048). In addition, nestling raised in larger broods had lower body condition (Tables 11 and S10).

Androgen-treated females showed a slight non-significant tendency to lay fewer eggs than controls, although clutch size was mainly affected by age and the specific breeding event at which they reproduce (Tables 10 and S9, all breeding event comparisons P < 0.023 where replacement > first > second clutches), increasing the number of eggs at older ages and decreasing it at the end of each breeding season. Egg volume, fledging success and other biometrical measures of nestlings, such as body size or condition, were not affected by female treatment (all P > 0.16). Nestling body weight and body condition were conditioned by female age and breeding event (Tables 10 and S9). As females grew older, their chicks had better weight and body condition, while the best scores were observed in chicks from replacement broods, as opposed to those developed in second broods (replacement > first > second broods, all P < 0.035). Chicks raised in larger broods had lower body weight and condition (Tables 10 and S9).

Fledging success and nestling body size were positively affected by female age (Tables 10 and S9). These two nestling variables were also affected by the breeding event in which chicks hatched (Tables 10 and S9), showing that fledging success was lower in second broods compared to first and replacement broods ($F_{2,200}$ = 1.73, P = 0.085 and $F_{2,217}$ = 2.81, P = 0.005, respectively) while body size was higher in replacement broods and lower in second broods (replacement > first > second broods, all P < 0.011).

Discussion

Unlike short-term effects of maternal hormones that have been empirically addressed by several studies (for a recent revision see Ruuskanen, 2015), the long-term consequences of such maternal effects remain largely speculative for most systems (Strasser and Schwabl, 2004; Daisley *et al.*, 2005; Eising *et al.*, 2006; Rubolini *et al.*, 2006; Tschirren *et al.*, 2007; Müller *et al.*, 2009; Müller and Eens, 2009). Here we show that an exper-

imental elevation of yolk androgen levels in a wild passerine exerted long-term consequences on multiple fitness-related traits at adulthood. We found that yolk androgens enhanced the development of a key ornamental plumage trait in this species, the length of the throat feathers. Yolk androgens exerted a detrimental effect on male survival, but only in those males hatching later in the season, whose survival was lower than controls. This could be associated to the adverse effect of yolk androgens on some components of the cellular immune response that was also detected. Despite this, the overall number of fledglings produced by males hatched from androgen-treated eggs was higher than that of controls during the first year, and this effect was reverted at older ages. In females, increased yolk androgens reduced clutch sizes, but increased the volume of the eggs laid. This resulted in larger nestlings when breeding conditions were harsher in contrast to the offspring of control females. There has been debate about whether long term maternal hormonal effects are the results of an organizational role of yolk androgen during development that modifies long-term profiles (Welberg and Seckl, 2001; Dufty *et al.*, 2002; Weaver *et al.*, 2004) or an indirect consequences of short-term effects drawn into adulthood (Carere and Balthazart, 2007; Groothuis and Schwabl, 2008). Regardless of this debate, mixes up ultimate and proximal levels of explanation (Krebs and Davies, 1997), this study shows sex-specific long-lasting fitness consequences of a prenatal exposure to high levels of maternal androgens, as well as transgenerational effects of exposure to maternal hormones.

Adult biometry

We found the enhancing effect of yolk androgens on a key secondary sexual plumage trait, the length of the throat feathers, in both males and females. These feathers represent a sexually selected trait in spotless starling males (Hiraldo and Herrera, 1974; López-Rull *et al.*, 2007; Gil and Culver, 2011), influencing female mating decisions (Aparicio *et al.*, 2001). Male starlings actively display these feathers while singing during courtship. Although this ornamental trait is less developed in females, there is evidence supporting a potential signalling role in females as well (López-Rull *et al.*, 2007, 2008). Therefore, our result points to an androgen-mediated mechanism by which also females could enhance offspring mating success. A similar enhancing role of maternal androgens on an adult feather ornament was found for the throat badge in male house sparrows (Strasser and Schwabl, 2004) or the nuptial plumage in male and female black-headed gulls (Eising *et al.*, 2006).

However, in contrast to plumage ornamentation, we found no effects of our experimental manipulation on adult size or condition. These results are consistent with those found in a previous study in which the same hormonal manipulation also had no effect on body size or condition of most of the nestlings, although androgen-treated chicks hatched in replacement clutches had a longer tarsus than controls (Muriel *et al.*, 2015a). However, the effect of yolk androgens may differ at different time scales. For example, it has been shown that tarsus length at fledging is significantly shorter in androgen-treated young compared to controls but it do not differ between treatments into adulthood, while body condition at fledging do not differ between treatments, although it is positively affected by yolk androgen treatment in males at adulthood (Ruuskanen *et al.*, 2012b). This is likely mediated by compensation in growth, a common pattern that has been shown to imply specific developmental costs (Metcalfe and Monaghan, 2001).

Testosterone levels, WBC counts and survival

Our results showed long-lasting consequences of yolk androgens on immunophysiological characteristics of the individual. The immunosuppressive effect of androgens (e.g., Folstad and Karter, 1992; Duffy *et al.*, 2000) has been repeatedly found in nestlings hatching from androgen-injected eggs (Groothuis *et al.*, 2005; Müller *et al.*, 2005; Navara *et al.*, 2006; Sandell *et al.*, 2009 but see Tschirren *et al.*, 2005; Andersson *et al.*, 2004). However, the very few long-term studies have only shown positive effects on humoral immunity (Tobler *et al.*, 2010) or no effect on cell-mediated immunity (Tobler *et al.*, 2010; Cucco *et al.*, 2008; Ruuskanen *et al.*, 2013). We found several differences in haematological composition that suggest a differential immune effect. In the first breeding season, androgen-treated males showed a tendency to have fewer circulating lymphocytes than controls, which could evidence an immunological cost since these cells have a key role in acquired immune responses due to its high antigenic specificity (Campbell and Ellis, 2007; Roitt *et al.*, 2001; Janeway *et al.*, 1999). In addition, based on differential WBC counts, we found that androgen-treated recruits tended have a higher chronic stress than controls, as defined by their higher H/L ratio (Gross and Siegel, 1983; Davis *et al.*, 2008). One possible explanation is that, mainly in their first reproductive year, the immunosuppressive effect of androgens led more susceptible recruits to any infection, where phagocytic heterophils (as components of nonspecific immunity) were elevated due to potential action in combating infection (Maxwell, 1993) and/or lymphocyte levels were low as part of the general response to stress (Dein, 1986). Accordingly, androgen-treated recruits also had elevated eosinophils relative to controls, perhaps due to activation of the innate immune defences because of a higher presence of parasites (Hawkey *et al.*, 1983; Campbell and Ellis, 2007; Roitt *et al.*, 2001).

Our experimental hormone manipulation strongly affected survival probabilities in males, with an interaction with timing of brood: whereas androgen-treatment males from late broods suffered reduced survival, the pattern was reversed in early clutches, with androgen-treatment males showing a trend for higher survival. Female survival, in contrast, was not affected by yolk hormone manipulations. These results are consistent with a similar study conducted in collared flycatchers (Ruuskanen *et al.*, 2012a), in which yolk androgen elevation lowered the local recruitment rate of male, but not female offspring (but see Rutkowska and Cichoń, 2006). The differential effect that we found in relation to timing of brood is in agreement with our data on the early development of these birds (Muriel *et al.*, 2015a). As nestlings, we found a similar brood effect on overall nestling survival, as well as reduced body condition in androgen-treated nestlings hatching from late broods. Late clutches are laid in the advanced breeding season, and experience higher chick mortality than in early broods, suggesting that the harsher environmental conditions experienced during late broods might reinforce the costs of high yolk androgen levels. This result is in agreement with the pattern of reduced yolk androgens allocation in second in comparison to first clutches (López-Rull *et al.*, 2010). Since nestling immunity has been shown to influence post-fledging recruitment (López-Rull *et al.*, 2011), the afore mentioned effects of yolk androgens on nestling immunity may have contributed to the lower survival rate of androgen-treated males hatched from late clutches.

Interestingly, the negative impact of yolk androgens on survival rate was restricted to males, whereas no effect was detected in females. This is likely due to sexual differences in susceptibility to androgens between males and females (see Badyaev, 2002), which could enhance the potential costs of these in the former, as found here for cellular immunological

parameters. In this sense, it is important to remark that we did not find any effect of yolk androgen manipulation on testosterone levels in the adulthood in any sex. This suggests that, rather than differences in hormonal state in adulthood, the observed pattern responds to long-term organizational effects of yolk androgen manipulation (Welberg and Sekl, 2001; Dufty *et al.*, 2002).

The reduced body condition at fledging of nestlings hatched from late clutches (Muriel *et al.*, 2015a) might have imposed a stronger handicap for survival in males as compared to females, as males face a stronger intrasexual competition for nesting places than females (as reflected by their lower success to establish as breeders in their first year of life) (Veiga *et al.*, 2002; this study). In addition, it is likely that males, being the larger sex, pay an extra developmental and physiological cost, as shown before in other species (Fargallo *et al.*, 2002; Müller *et al.*, 2003). The contrasting reproductive roles and ecological trade-offs among sexes likely reinforce the observed sex-dependent costs of yolk androgens.

In addition to higher mortality, the lower survival of androgen-treated males from late broods could be explained by a higher dispersal probability of this group (see Tschirren *et al.*, 2007). However, the fact that the negative impact of increased yolk androgens on survival was observed only in males hatched from late broods makes this unlikely, as there is wide evidence that late clutches often actually result in lower fledgling survival and later recruitment rate in birds (e.g. Lack, 1968; Price *et al.*, 1998). Our results also showed that overall survival rate decreased with increasing age, as expected. Female lifespan was shorter than that of males, probably due to their higher reproductive investment (e.g. Jimeno *et al.*, 2014) and that first-time breeders often pay a higher survival cost if they attempt to breed at a young age (Williams, 1957; Pyle *et al.*, 1997).

Reproduction

The overall number of breeding attempts per year, successful or not, increased as individuals grew older, probably due to the higher quality and experience of the survivors, which probably increased their resource-holding potential. However, both female and male recruits that hatched from early clutches performed better than those coming from late clutches. This was likely due to the superior body condition of chicks from early broods (Salaberria *et al.*, 2014; Muriel *et al.*, 2015a), together with the more favourable environmental conditions that they encountered at fledging (Ilyina *et al.*, 2013). Despite the higher survival rate of males in their first year of life, very few managed to breed, whereas females were more successful overall as first year breeders. This is a well-known pattern in other similar starling species (Feare, 1984).

Overall, the reproductive success (number of fledglings produced, considering only those broods where at least one fledged) of starlings improved with age, probably as a result of improved foraging, breeding skills and resource-holding potential (e.g., see Mauck *et al.*, 2004). The general pattern for birds is usually an inverse U-shaped curve of age-related changes in breeding success (Clutton-Brock, 1984; Curio, 1983; Newton, 1989), our study only covered the first three years of life of our cohort. Given that starlings can live up to 8 or 9 years (Veiga and Polo, 2011), our time frame seems too short to detect a hypothetically quadratic pattern. Regarding the effects of our hormonal manipulation, we found sex-specific and context-dependent effect of yolk androgens. Androgen-treated males hatched in late clutches were reproductively less successful than controls or those androgen-treated males hatched in early clutches. The underlying proximate

mechanisms explaining this pattern are likely similar to those described for survival rate (see above). Androgen-treated males hatching from late broods that survived and managed to reproduce still showed a reduced performance possibly because of the detrimental combination of high androgens and harsh environmental conditions in late clutches (Muriel *et al.*, 2015a).

Breeding parameters

We found that, during their first breeding season, androgen-treated females reduced clutch size and increased the average volume of the eggs laid with respect to control. These results are consistent with previous evidence for the same species (Veiga and Polo, 2008; but see López-Rull and Gil, 2009), as well as for zebra finches (Rutkowska *et al.*, 2005), which found that females with testosterone implants laid fewer eggs per year, but eggs were heavier than those laid by control females. These studies suggested a trade-off between clutch size and egg size which is in line with the general life-history trade-off between size and number of offspring (Stearns, 1992). We hypothesise that elevated circulating T might stimulate higher investment in eggs but at the expense of laying fewer eggs. Although we did not find differences in pre-breeding plasma testosterone between control and androgen-treated females, levels might have changed later during ovulation. Thus, our data would support an organizational role of early exposure to testosterone, whereby androgen-treated females would prioritize quality versus quantity (Williams, 2001). Importantly, we have recently shown in this species that egg size exert positive effects pervasive throughout the nestling period (Muriel *et al.*, 2013). Consistently with this, we found that nestlings of these androgen-treated females were indeed larger at fledging in second broods. However, these effects of yolk androgens on first female reproduction vanished in the following years. Interestingly, another study has found that females treated with an anti-oestrogen as adults produce larger clutches with smaller eggs (Williams, 2001). The mechanism behind these changes is difficult to unravel, since the interplay between biomolecules (e.g. enzymes) and steroids is complex and tissue-dependent, but this evidence suggests that an important life-history trade-off could be manipulated by hormonal exposure.

In agreement with evidence from a similar study conducted in a wild breeding population of collared flycatchers (Ruuskanen *et al.*, 2012a), our experimental increase in yolk androgens in females did not affect the fledging success of their offspring. In our study population the number of fledglings decreases over the breeding season (e.g., López-Rull *et al.*, 2011; Muriel *et al.*, 2015a), and increases as females reach 2-3 years of age (Polo and Veiga, 2006). However, in the case of males, we found an age-specific effect of treatment on fledging success. Androgen-treated males produced more fledglings than controls at their first and second year of age, but but the pattern tended to reverse at their third year. One possible explanation for this result is that yolk androgens promoted a higher investment of treated males in reproduction at their first years of life, which would be detrimental at older ages (this is reminiscent of a pattern of “live fast, die young” pace of life, e.g. Hamilton and Zuk, 1982; Balenger and Zuk, 2014). The mechanism could actually be due to androgen-treated males attracting higher quality females, thanks to the enhanced development of their throat ornamental feathers (Aparicio *et al.*, 2001). In addition, literature shows females typically invest higher levels of resources in the offspring of attractive males (e.g. Gil *et al.*, 1999; Alonso-Alvarez *et al.*, 2012). Given that throat feather length naturally increases with age (Hiraldo and Herrera, 1974), the advantage conferred by androgen levels would be noticeable mostly at early ages,

but would be offset as males grow older, thus explaining the age-by-treatment pattern detected.

Concluding remarks

This study complements previous results on this avian population, where we highlighted the adaptive value of the short-term and context-dependent effects of yolk androgens (Muriel *et al.*, 2015a). By following surviving offspring in the field for three years we were able to show that yolk androgens also exert long-lasting effects on key life history traits, modifying trade-offs among them, as well as inducing transgenerational changes. These long-term effects often emerged in a sex-specific way, showing that the developmental processes that androgens modify are sex-specific. Long-term effects of yolk androgens in males had implications for sexual selection, favouring large secondary sexual traits. However the potential subsequent benefits in terms of mating success benefits might be balanced by a decrease in survival in those birds experiencing tough conditions during the nestling period (Muriel *et al.*, 2015a). In females, yolk androgens did not exert long-term effects on survival, but had a greater impact on its reproductive physiology and fertility, particularly in their first year of life. These findings give support the idea that maternal androgen allocation is likely limited by a sexual conflict in which the reproduction of sons and daughters is affected in different ways by the same phenotypic modification, as well as by transgenerational effects on male and female offspring (Rubolini *et al.*, 2007). Therefore, this study supports the existence of phenotypic plasticity mediated by hormone-related maternal effects which entails direct effects on offspring fitness (e.g. Mousseau and Fox, 1998a; Badyaev and Uller, 2009). Since males and females have different reproductive roles and ecological trade-offs, future studies should explore in more detail the sex-specific effects of yolk androgens on mating behaviour and lifetime breeding success. Understanding the lifetime sex- and context-dependent effects of yolk androgens is essential to fully appraise the adaptive importance of the hormone mediated maternal effects as well as the potential constraints shaping their contribution to individual fitness.

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Supplementary Table S8. Summary of final repeated-measures mixed models showing the estimates and standard error (estimate ± SE) of all the variables affecting adult body size, body weight, body condition and ornamental feather length. Models were run using Proc Mixed with Satterthwaite correction to adjust the degrees of freedom. Fixed factors were coded as treatment (control and androgen treated) and sex (male and female). Age was measured in years.

	Body size	**Body weight**	**Body condition**	**Ornamental feather length**
Independent variable	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***
Intercept	-1.404 ± 0.373	88.19 ± 2.343	1.155 ± 0.434	17.375 ± 0.645
Treatment (control)	–	–	–	-1.311 ± 0.493
Sex (male)	0.913 ± 0.142	4.325 ± 0.647	–	10.422 ± 0.479
Age	0.160 ± 0.057	0.866 ± 0.406	–	3.308 ± 0.267
Capture date	–	0.053 ± 0.016	0.010 ± 0.004	–

Supplementary Table S9. Summary of final repeated-measures mixed models showing the estimates and standard error (estimate ± SE) of all the variables affecting clutch size, egg volume, and offspring (F1) tarsus length, body condition and fledging success of female recruits, both for the first breeding season as all breeding seasons pooled. Models were run using Proc Mixed with Satterthwaite correction to adjust the degrees of freedom. Fixed factors were coded as treatment (control and androgen treated) and breeding event (first, replacement and second brood). Age was measured in years.

		Clutch size	**Egg volume**	**F1 Tarsus length**	**F1 body condition**	**Fledging success**
	Independent variable	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***
First breeding season (2012)	Intercept	3.295 ± 0.216	6451.4 ± 72.51	29.74 ± 0.487	0.9833 ± 0.3847	0.1231 ± 0.07499
	Treatment (Control)	0.434 ± 0.198	205.3 ± 99.95	-2.252 ± 0.691	–	–
	Breeding event (First)	0.745 ± 0.248	–	0.407 ± 0.501	1.2700 ± 0.3365	0.2907 ± 0.09583
	Breeding event (Replacement)	1.054 ± 0.253	–	0.312 ± 0.572	1.1478 ± 0.3711	0.3035 ± 0.09903
	Treat × Breeding event (First, Control)	–	–	2.135 ± 0.712	–	–
	Treat × Breeding event (Replacement, Control)	–	–	2.449 ± 0.783	–	–
	Brood size	–	–	–	-0.535 ± 0.103	–
All breeding seasons (2012-2014)	Intercept	3.104 ± 0.213	–	29.17 ± 0.244	-2.315 ± 0.950	-2.015 ± 0.509
	Treatment(Control)	0.229 ± 0.132	–	–	–	–
	Breeding event (First)	0.467 ± 0.140	–	0.347 ± 0.142	0.699 ± 0.155	0.445 ± 0.257
	Breeding event (Replacement)	0.844 ± 0.179	–	0.783 ± 0.194	1.056 ± 0.204	0.909 ± 0.324
	Age	0.553 ± 0.084	–	0.249 ± 0.089	0.264 ± 0.094	0.985 ± 0.159
	Brood size	–	–	–	-0.229 ± 0.059	–
	Egg volume	–	–	–	0.001 ± 0.001	–

Supplementary Table S10. Summary of final repeated-measures mixed models showing the estimates and standard error (estimate ± SE) of all the variables affecting male breeding parameters (offspring -F1- tarsus length, body condition, and fledging success) for all breeding seasons. Models were run using Proc Mixed with Satterthwaite correction to adjust the degrees of freedom. Fixed factors were coded as treatment (control and androgen treated) and breeding event (first, replacement and second brood). Age was measured in years.

		F1 Tarsus length	**F1 body condition**	**Fledging success**
	Independent variable	***Estimate* ± *SE***	***Estimate* ± *SE***	***Estimate* ± *SE***
All breeding seasons (2012-2014)	Intercept	29.021 ± 0.348	-120.6 ± 16.79	1.497 ± 0.860
	Treatment (Control)	–	–	-2.751 ± 1.356
	Breeding event (First)	-0.112 ± 0.151	4.439 ± 0.815	–
	Breeding event (Replacement)	0.448 ± 0.224	8.156 ± 1.435	–
	Age	0.375 ± 0.132	4.245 ± 0.848	0.546 ± 0.358
	Treat × Age	–	–	1.139 ± 0.561
	Brood size	–	-0.575 ± 0.315	–

INTEGRATIVE DISCUSSION

The general aim of this Thesis is to study the importance of maternally derived androgens on offspring performance, which is essential to evaluate their role in mediating fitness differences between different phenotypes. Hormone-mediated maternal effects, such as those derived from exposure to yolk androgens during early development in birds, may exert variable effects on several offspring life-history traits (Groothuis *et al.*, 2005a; Gil, 2008; Ruuskanen, 2015), but their adaptive value is at present unclear. In short, several studies have shown that yolk androgens improve nestling growth and survival (Schwabl, 1993; Eising *et al.*, 2001; Pilz *et al.*, 2004), while other studies have found the opposite effect (Sockman and Schwabl, 2000; Rubolini *et al.*, 2006). In line with the inconsistent effects on growth, the evidence of a negative effect of androgens on immune response is mixed: although most studies reported a clear immunosuppressive effect (Müller *et al.*, 2005; Navara *et al.*, 2005; Sandell *et al.*, 2009), in other cases androgen injection did not influence the immunity (Tschirren *et al.*, 2005; Rubolini *et al.*, 2006; Pitala *et al.*, 2009) and even exerted a positive effect (Navara *et al.*, 2006). Similarly, the few studies that have analysed the long-lasting effects of yolk androgens have reported both positive (Strasser and Schwabl, 2004; Eising *et al.*, 2006) and negative effects (Rubolini *et al.*, 2006; Bonisoli-Alquati *et al.*, 2011) on adult sexual ornaments. Through the different chapters of this Thesis, we have assessed several factors that could explain, to some extent, the above mentioned mixed evidences of the effects of yolk androgens, namely: the particular effects of each specific yolk androgen, the existence of linear and non-linear dose-dependent effects, and the context-dependent balance between costs and benefits of these hormones. Also, in order to evaluate the fitness consequences of maternal androgens, we assessed their long-term effects on individual phenotype, survival and breeding output.

In Chapter I, we tested the differential effect of physiological *in-ovo* injections of two different androgens (T and A4) and their combination in embryo and nestling growth in the spotless starling. We found different effects of both androgens on embryonic and nestling development, suggesting that their effects vary depending on the developmental phase considered and on specific androgenic pathway (Navara *et al.*, 2005; Rubolini *et al.*, 2006; von Engelhardt *et al.*, 2009). Duration of the embryonic period was increased by T and less so by A4, but not by the combination T+A4. Body condition was reduced in all experimental treatments where A4 was present, particularly so in the combination T+A4. The combination T+A4 showed a strong reduction in male growth, also limited to that early period, since differences disappeared at the end of nestling period. Cell-mediated immune responsiveness, at least at the end of the nestling period, was only marginally affected by the interaction between treatment and sex. These patterns suggest that in this species, T has a stronger influence during embryo development than A4, whereas during nestling development the capacities of both androgens to influence growth are similar. The combination T+A4 showed non-additive effects, suggesting some kind of inhibition between

the two androgens. A similar pattern was found in a comparative analysis (Schwabl *et al.*, 2007), in which species-specific differences in T were more strongly correlated with embryo than with nestling developmental periods. Our study therefore reveals that yolk A4 and yolk T are associated with different biological consequences (see also Hegyi *et al.*, 2011; Tschirren *et al.*, 2014), suggesting a complex picture of sex and age-dependent effects of both yolk androgens.

Another important issue that has been overlooked in previous research is the likely non-linear nature of hormone effects. Since complex dose-response patterns may shift the balance from benefits to costs (Navara *et al.*, 2005; Bhasin *et al.*, 2001), in Chapter II we experimentally tested the differential effect of physiological in-ovo injections of the naturally-occurring mixture of yolk T and A4 at three different doses on offspring development and immune responsiveness. We found that yolk androgens show a non-linear dose-response pattern for several traits. Thus, androgens had a stimulatory effect on hatching body mass and nestling skeletal growth, but maximum values were found at intermediate doses, whereas our highest dose resulted in a decrease. Our results are consistent with other studies which have found that prenatal exposure to androgens can have positive effects on growth (Eising *et al.*, 2001; Schwabl, 1996; Bentz *et al.*, 2013; but see Chapter I), probably mediated by an increase in metabolic rate (Tobler *et al.*, 2007). In addition, our finding on nestling growth is consistent with a similar trend described by Navara and co-authors (2005) who found that moderate levels yolk T, but not the high dose, tended to have a stimulatory effect on nestling size. However, the opposite *U-shaped* effect was found on nestling body mass. Low- and intermediate-doses may derive a weight advantage from fast hatching, but this difference may not be enough to offset the costs and increase mass accordingly. This pattern may be explained if body mass and skeletal growth trade-off against each other. Similarly, in Chapter I we found a reduction in body condition of T + A4 nestling group (1 SD). We also detected linear negative and positive effects on embryonic development period (Eising *et al.*, 2001; Eising and Groothuis, 2003; but see Chapter I), and nestling gape width (Müller *et al.*, 2007), respectively. In Chapter I, no differences were observed on embryonic developmental period from eggs injected with T + A4, but in that case the injected dose was smaller (only 1 SD of the population mean). During this initial period of nestling development, chick survival strongly depends on attracting parental feedings (Gil *et al.*, 2008), so it makes sense that gape growth should be particularly labile during this time. Our results suggest differential tissue responsiveness to yolk androgens, which may result in compromises in maternal allocation to produce adapted phenotypes. Also, from an adaptive perspective, our results shed some light to the observed variability in maternal androgen allocation. Given the suboptimal cost/benefits balance at high androgen concentrations, females would not be subject to directional selection to allocate more androgens to the egg yolk, but this maternal transfer would rather be fitted to individually optimal –not necessarily highest- levels. Indeed, as found in Chapter IV, variation in maternal androgen allocation to eggs could be explained as the result of context-dependent optimal strategies to maximize offspring fitness. Maternal accurate modulation of yolk androgens could therefore be an adaptive strategy to handle the context- and dose-dependent effect of androgens (e.g. Martinez-Padilla *et al.*, 2010; Martínez-Padilla *et al.*, 2014). Given the non-linear dose-response patterns found, future investigations should carefully consider the natural variability of yolk androgen levels in their study population, and hence adjust hormonal manipulations within the physiological range, since the balance of costs

and benefits may strongly differ depending on concentration.

As said above, although Chapter II showed how nestlings benefit from increased yolk androgens in terms of enhanced growth, high levels of yolk androgens can also involve costs (Groothuis *et al.*, 2005b). In fact, the accelerated growth induced by yolk androgens, may lead suppression of immune responsiveness because of the trade-off between accelerated growth and immune function (Groothuis *et al.*, 2005b; Müller *et al.*, 2005; Sandell *et al.*, 2009). However, very few avian studies have examined dose-dependent immunological consequences of yolk androgens (e.g. Norton and Wira, 1977; Navara *et al.*, 2006). Unlike for nestling growth, in Chapter III we did not detect a complex dose-response pattern for immunological parameters. By contrast, we showed that experimentally increased yolk androgens led to sex-specific immunosuppression of two measures of innate and adaptive axes of the immune system. Both cell-mediated immune response and lysozyme activity decreased with increasing androgen injections in the egg in the case of male nestlings, whereas there were no effects on females. These results are consistent with Chapter I, where male chicks that hatched from yolk androgen-treated eggs suffered a slight suppressive effect on cell-mediated immune responsiveness. Since male and females chicks in our study species have similar levels of testosterone (Gil *et al.*, 2008; Müller *et al.*, 2007), their larger immune susceptibility should arise by differences in hormone metabolism or function (Duffy *et al.*, 2000; Fargallo *et al.*, 2002; Møller *et al.*, 1998; Moreno *et al.*, 2001). We found no effects of the experimental treatment on humoral immunity (haemolysis - agglutination assays), but these measures were negatively correlated to cell-mediated response, suggesting negative covariance among different branches of the immune system (Forsman *et al.*, 2008; Palacios *et al.*, 2007). Thus, our results show that in our study species, yolk androgens induce immunosuppression in some axes of the immune system in male nestlings. Given the complex nature and interconnection of the different axes of the immune system, this does not allow us to predict how other components of the immune response could be affected by yolk androgens. Likely, yolk androgens may prime the development of some components of the immune system contrasted ways (Sandell *et al.*, 2009); showing sex- and context-specific effects (Chapter IV).

Apart from the effects of the dose-dependent effects addressed in Chapters II and III, the balance between costs and benefits of androgen allocation is expected to be affected by the environmental context where the nestling is growing. However, such hypothetical context-dependent effect of yolk androgens on offspring physiology has been scarcely explored. In Chapter IV we investigated whether the effects of yolk androgens on nestling development and immunity changed depending on the breeding attempt, as the environmental conditions become harsher (Salaberria *et al.*, 2014) and parental energetic reserves are gradually reduced as the breeding season advances (Verhulst and Tinbergen, 1991; Reed and Clark, 2011). Both in first and replacement broods, the embryonic development period was shorter for androgen-treated chicks than controls, but there were no differences in second broods. These results are consistent with those found in Chapter II, although in that case nestling monitoring was not considered in second broods. In replacement broods, androgen-treated chicks were heavier and larger than those hatched from control eggs, but this effect was not observed in the other breeding attempts. Again, this is consistent with Chapter II where chicks hatched from eggs injected with the same androgen dose used here where bigger than controls. A possible explanation for this particularly high sensitiveness of replacement broods to androgen injections

is that females laying a replacement clutch suffered stronger resource limitations due to their double laying effort (Hipfner *et al.*, 1999; Gasparini *et al.*, 2006; but see Gasparini *et al.*, 2007), and it is possible that yolk androgen injection may have compensated to some extent this constraint, bolstering nestling development of experimental clutches. By contrast, androgen exposure reduced survival with respect to controls only in second broods. Regarding immune function, we detected marginally significant trends for androgen treatment to activate two important components of innate and adaptive immunity (IL-6 and Ig-A levels, respectively), suggesting that an inflammatory process is taking place in chicks hatched from androgen-treated eggs. Also, cell-mediated adaptive immunity, measured as proliferation of T-cells, was higher in androgen-treated chicks than in controls in first broods, whereas the opposite pattern was found in second broods. Unfortunately, we cannot directly compare the patterns found for cell-mediated immunity in Chapter IV with those reported in Chapter III, as in the latter we used a different immunological test that not only involves proliferation of T-cells but also the secretion of proinflammatory cytokines, basophils, heterophils, and macrophages (Martin *et al.*, 2006; Salaberria *et al.*, 2013), and thus involves both innate and adaptive components of the immune system (Bílková *et al.*, 2015; Stadecker *et al.*, 1977). In any case, the results of Chapter IV suggest that, in a context in which breeding conditions become harsher with the progress of the season, decreased survival of androgen-treated chicks may be explained, at least partly, by a greater susceptibility of these nestlings to disease (Folstad and Karter, 1992; Buchanan *et al.*, 2003; Roberts *et al.*, 2004; Navara *et al.*, 2005; but see Evans *et al.*, 2000; Navara *et al.*, 2006). This difference in the effects of yolk androgens on first vs. second broods could be mediated, in the last term, by differences in environmental food availability, as it is known that nutritional status influences immune responses (Norris and Evans, 2000; Zuk and Stoehr, 2002). The seasonal variation in maternal androgen allocation to eggs detected in this and other species (López-Rull *et al.*, 2010; Vergauwen *et al.*, 2012) could therefore be an adaptive adjustment resulting from an optimal context-dependent optimal strategy to maximize offspring fitness.

In Chapters I to IV we addressed short term hormone-mediated maternal effects on developmental and physiological traits of offspring. But, do yolk androgens exert significant effects on fitness-related traits at adulthood? Relatively few studies have analysed the long-term effects of yolk androgens on individual phenotype and performance. In Chapter IV we experimentally manipulated yolk androgen levels on 497 clutches, subsequently monitoring adult phenotype, immune function, recruitment and breeding output in the following three breeding seasons (Chapter V). We found that yolk androgens enhanced the development of a key ornamental plumage trait in this species at adulthood, the length of the throat feathers. Therefore, our result points to an androgen-mediated mechanism by which females could enhance offspring mating success. However, these positive effects of yolk androgens were offset by costs in a context- and sex-specific way. Thus, yolk androgens exerted a detrimental effect on male recruitment, but only in those males hatching later in the season, whose recruitment was lower than controls. In the analysis of growth of these birds as nestlings in Chapter IV, we showed the same context-dependent effect in overall nestling survival, as well as a reduced body size and weight in the surviving nestlings hatching from androgen treated clutches. This reduction in male survival could be associated with the adverse effect of yolk androgens on some components of the immune function that was also detected (Chapter III and IV). It could be possi-

ble that the poorer body condition at fledging of nestling hatched from androgen-treated eggs in late clutches (Chapter IV) might have imposed a stronger handicap for survival in males as compared to females, since males face a stronger intrasexual competition for nesting places than females (Veiga *et al.*, 2002; Chapter V). The contrasting reproductive roles and ecological trade-offs among sexes likely underlie the observed sex-dependent costs of yolk androgens. In addition, as discussed in Chapter III, it is coherent that males pay an extra immunological cost, as has often been shown in several studies across a diverse range of species (e.g. Fargallo *et al.*, 2002; Lobato *et al.*, 2008; Müller *et al.*, 2003). Despite this, the number of chicks fledged by androgen-treated males was higher than that of controls at first years, although this effect was reverted at later in life. One possible explanation for this result is that yolk androgens promoted a higher investment of treated males in reproduction at their first years of life, which would be detrimental at older ages (e.g. Hamilton and Zuk, 1982; Balenger and Zuk, 2014). Alternatively, it is also possible that androgen treated males resulted more attractive to females (Aparicio *et al.*, 2001), due to their larger development of feather ornaments (see above). This would result in higher chances to mate with higher quality females that would in turn invest more resources in current reproduction (Gil *et al.*, 1999; Sheldon, 2000; Harris and Uller, 2009). Given that throat feather length naturally increases with age (Salaberria, 2011), the advantage conferred by androgen levels would be noticeable mostly at early ages, but would be offset as males grow older, thus explaining the age-by-treatment pattern detected. On the other hand, we found that, during their first breeding season, androgen-treated females reduced clutch size and increased the average volume of the eggs laid with respect to controls. Such trade-off between clutch size and egg size is in line with the general life-history trade-off between size and number of offspring (Williams, 2001). Thus, our data could support an organizational role of early exposure to androgens, whereby androgen-treated females would prioritize quality versus quantity. Beyond transmission of maternally-derived substances, avian maternal effects could be also related with egg size and quality (Rubolini *et al.*, 2011). In this regard, as we have shown in Chapter I, egg size exerts long-lasting positive effects throughout the nestling period (Muriel *et al.*, 2013). Consistently with this, we found that nestlings raised by these androgen-treated females were indeed larger at fledging in second clutches. Taken together, the results of Chapter V demonstrate that maternally derived androgens can modify offspring adult phenotype, affecting key life-history traits, and also inducing transgenerational changes.

To conclude with, our findings support the importance of hormone-mediated maternal effects on individual phenotypic plasticity, as well as how these effects may vary depending on additional factors, such as embryo sex, developmental phase, type and concentration of androgens, and environmental context. Variations in yolk androgen levels exert both short- and long-term effects on different life-history traits that ultimately impact on offspring fitness. Thus, this Thesis highlights the implication of maternally derived androgens on offspring performance, adding support to their potential role in adaptive processes.

Outstanding questions

Since our own results reveal that yolk A4 and yolk T are associated with different biological consequences, future studies in hormone-mediated maternal effects should explore separately their sex- and context-specific effects in as many life stages as possible. This would be important to improve our understanding of the adaptive significance of each yolk

androgen as physiological mediator and modulator of life history trade-offs. For instance, their concentrations could not only affect differentially to sons and daughters because of their sex-specific sensitivity, but their effects can also differ depending on the context where the offspring develop. Also, we highlight the necessity of further research on the proximate physiological and molecular mechanisms underlying hormone deposition in the egg yolk, the embryonic utilization of yolk androgens and the pleiotropic actions of these maternal hormones, which may also differ among each specific yolk androgen. This aspect warrants particular consideration since mechanisms can determine and limit, via physiological trade-offs, the adaptive potential of yolk androgens. Also, a better understanding of these physiological mechanisms may shed light on the ability of androgens to influence the expression of suites of correlated traits (Groothuis and Schwabl, 2008).

Besides their implications for a better understanding of the effects of yolk androgens on offspring development, our results beg the question of why there is individual variation in androgen allocation among females. The answer may lay not only in the potential costs for offspring in the short- or long-term, as shown here, but also in just how costly is for females the mobilization and allocation of yolk androgens per se. To date, few studies have addressed the mechanistic aspects of yolk hormone deposition, to what extent can females strategically adjust androgen deposition, and the potential physiological costs or limitations of this process for mothers. These are long standing questions since the discovery of androgens in the egg yolk, but our knowledge on these aspects is still very limited. Until these questions are solved, we will not have a complete picture of the trade-offs regulating androgen-mediated maternal effects.

It is known that androgens may be beneficial to the offspring, but these can also lead to immunological costs. However, since our results show a negative covariance among different branches of the immune system, future studies addressing the effects of maternal androgens on immune response should consider a wider range of immunological assays covering as many branches of the innate and adaptive immune system as possible. Beyond immunological costs, yolk androgens may lead metabolic costs that could be related to an overproduction of free oxygen radicals and, thus, an increased risk of oxidative stress (Halliwell and Gutteridge, 2007). This can impose an extra cost to developing offspring, but also constitute an important parameter to be considered in the trade-offs involving growth and immunity (e.g. Hall *et al.*, 2010; Moreno-Rueda *et al.*, 2012). Further physiological studies are needed to investigate their relationship with hormone-mediated maternal effects since they could be the key to understand the constraints imposed by yolk androgens to male offspring. Our findings suggest that prenatal environmental factors, may act as maternal cues to adjust the yolk androgen levels to each breeding context, possibly to maximize offspring fitness. However, this conclusion comes from the natural variation of the environmental conditions as the breeding season progresses, without an experimental manipulation of environmental variables. Therefore, future experimental studies should address what specific environmental variables modulate the effects of yolk androgens, which could be very useful for understanding the adaptive relationship between hormone-mediated maternal effects and environmental context.

Although most previous experimental studies on yolk androgens have focused on their effects on early life stages, these maternal hormones do exert long-lasting consequences on offspring life history traits, and further involve transgenerational changes. However, although our work consistently support the existence of long-lasting hormone-

mediated effects, future experimental studies are required to fully assess the relationship between prenatal exposure to androgens and lifetime breeding success, testing the relative contribution of androgens to different parameters (i.e. life expectance, timing of reproduction, overall investment, breeding strategies, etc). In this line, since males and females have different reproductive roles and ecological trade-offs, future studies should explore in more detail the sex-specific effects of yolk androgens on mating behaviour. Thus it could be possible that yolk androgens may have mediated the evolution of sex differences in allocation of reproductive effort to mate attraction and parental care, even affecting rates of extra-pair fertilizations. However, may be difficult to speculate about the mechanism by which yolk androgens affects adult behaviour unless we know about its effects on the dynamics of the endocrine responses, hormone receptors and metabolising enzymes (von Engelhardt, 2004).

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CONCLUDING REMARKS

1. The two main androgens found in avian yolks, testosterone (T) and androstenedione (A4), exert different effects during early development. In our study species, the spotless starling (*Sturnus unicolor*), T has a stronger influence during embryo development than A4, whereas during nestling development the capacities of both androgens to influence growth are similar. However, T and A4 show non-additive effects, suggesting either some kind of inhibition between them. Our results reveal that yolk A4 and yolk T are associated with different biological features, and underline the necessity of further research on the differential metabolism and action of egg androgens.

2. Beyond effects of yolk androgens, we found a strong effect of egg size and laying order throughout nestling development. The influence of egg size on nestling size and condition was detected even at the end of the nestling period, which highlights the importance of female investment in this trait on subsequent nestling development. On the other hand, we cannot discard that this trait is correlated with other aspect of female quality or investment that was not measured in the study.

3. Yolk androgens show complex dose-response effects during early development, including both linear and non-linear responses for different nestling traits. This highlights the importance of accurately adjusting the dose to the natural yolk androgen levels of the species in manipulative studies, as well as considering dose-dependent effects when interpreting the results. More importantly, this suggests that the balance of cost and benefits is a subtle one, and that higher androgens levels are not always beneficial.

4. For some traits, yolk androgen effects were mostly detected at earlier phases of the nestling period, whereas for others the effect was stronger in later stages, thus illustrating the variability in responsiveness to the hormone across traits.

5. Experimentally increased yolk androgens result in sex-specific immunosuppression: increasing levels of yolk androgens exert a negative impact on of two measures of innate and adaptive axes of the immune system (lysozyme activity of the plasma and cell-mediated immunoresponsiveness) in male nestlings but not in females. These results support the existence of immunosuppressive costs associated to yolk hormones.

6. We detected a negative covariance among different branches of the immune system. Future studies on the effects of maternal androgens on immune response should consider a wider range of immunological assays in order to cover as many branches of the innate and adaptive immune system as possible.

7. The effect of yolk androgens on both pre- and post-hatching development and immune function is context dependent: increased yolk androgens accelerated embryon-

ic development and improved lymphocyte proliferation (a measure of adaptive immunity) in early clutches, but reduced nestling survival and lymphocyte proliferation in clutches laid at the end of the breeding season, when environmental conditions are harsher. This pattern confirms the adaptive value of seasonal decline in egg yolk androgen levels observed in this and other avian species. Ultimately, variation in maternal androgen allocation to eggs could be explained as the result of context-dependent optimal strategies to maximize offspring fitness.

8. Yolk androgen levels not only modulate individual development during the nestling period, but exert long term effects on key life-history traits in a sex-specific way. Yolk androgens enhance the development of a plumage sexual ornament in this species (the length of throat feathers). However, in males, they also impair cellular immune function and their subsequent survival rate and breeding success of birds hatched in late clutches. Therefore, long-lasting effects of yolk androgens in males could be framed in the general trade-off between survival and reproduction.

9. In females, yolk androgens do not exert long-term consequences on survival, but influence the trade-off between quality and quantity of propagules: high yolk androgens levels led females to produce larger eggs and shorter clutches and larger eggs at adulthood, which resulted in increased offspring size at the F1. Yolk androgens can therefore induce transgenerational plastic responses in the phenotype of individuals.

10. Our findings support the importance of hormone-mediated maternal effects on individual phenotypic plasticity. Variations in yolk androgen levels exert both short- and long-term effects on different life-history traits that ultimately impact on offspring fitness. Thus, this Thesis confirms the implication of maternally derived androgens on offspring performance, adding support to their role in mediating evolutionary change.