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MATERNAL EFFECTS IN BIRDS – THE ECOLOGICAL AND EVOLUTIONARY SIGNIFICANCE OF YOLK ANDROGENS AND OTHER EGG COMPONENTS

by

Suvi Ruuskanen

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UNIVERSITY OF TURKU
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From the Section of Ecology, Department of Biology, FI-20014 University of Turku, Finland

Supervised by

Dr. Toni Laaksonen
Finnish Museum of Natural History
University of Helsinki
Helsinki, Finland

Reviewed by

Dr. Tobias Uller
Department of Zoology
Oxford University
Oxford, UK

Dr. Esa Koskela
Department of Biological and Environmental Science
University of Jyväskylä
Jyväskylä, Finland

Examined by

Prof. Jan-Åke Nilsson
Animal Ecology
Department of Biology
Lund University
Lund, Sweden

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IV Ruuskanen, S., Helle, S., Ahola, M., Adamczyck, F., Möstl, E. & Laaksonen, T. Digit ratios are neither a marker of maternally-derived androgen exposure nor associated with reproductive traits or exploratory behavior in a wild bird population. (Submitted manuscript)

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1. INTRODUCTION

Offspring phenotype is the product of genes transferred from parents and the environment experienced by the offspring. In addition, parents affect the environment of their offspring for example by providing resources and parental care. Maternal effects are referred to when maternal phenotype affects offspring phenotype (Bernardo 1996a, b; Mousseau and Fox 1998a, b). In a broad sense, maternal effects can be solely environmental, genetic, or genes and the environment may interact (indirect genetic and environmental effects, Mousseau and Fox 1998a; Wolf and Wade 2009). Previously maternal effects have been treated as a source of confusion in determining quantitative genetic parameters precisely, but it is now widely appreciated that maternal effects are ubiquitous and can play a very important role in evolutionary processes (Bernardo 1996a, b; Rossiter 1996; Mousseau and Fox 1998a, b; Galloway 2005; Räsänen and Kruuk 2007; Marshall et al. 2008; Badyaev 2008; Uller 2008; Badyaev and Uller 2009). Maternal effects can be seen as a source of adaptive trans-generational phenotypic plasticity: via the resources and the growth environment parents provide for their young, parents can translate their environmental or social experience into adaptive variation in the offspring, increasing offspring fitness (Mousseau and Fox 1998a). The adaptive nature of maternal effects has been discovered in various animal and plant taxa (reviewed by Bernardo 1996a; Rossiter 1996; Mousseau and Fox 1998a, b; Lindström 1999; Metcalfe and Monaghan 2001; Galloway 2005; Groothuis et al. 2005b; Hasselquist and Nilsson 2009). However it must be acknowledged that not all maternal effects are adaptive from the offspring perspective, but can be adaptive from the maternal perspective, in the context of life-time reproductive output: A decrease in offspring fitness may be favored to increase the mother's future reproductive success and survival (Trivers 1974; Godfray 1995; Marshall and Uller 2007; Uller 2008). Maternal effects are thus subject to different selection pressures from both the parent and the offspring side, which will often lead to conflicts.

1.1. Avian egg as a model

Avian egg is an excellent model to study maternal effects, as all the material resources the embryo has for growth and development are incorporated in the eggs (compared to mammalian systems), and the egg components can be fairly easily measured and even manipulated (Groothuis and von Engelhardt 2005). In addition to nutrients (proteins and lipids) for the growing embryo, eggs contain several components important for the development of the young. These include carotenoids, which are antioxidants that reduce lipid peroxidation in the embryo and enhance immune function (Surai and Speake 1998; Blount et al. 2000). Eggs also contain maternally-derived immunoglobulins which provide the primary form of humoral immune defence for the offspring, as underdeveloped young cannot synthesize these (reviewed by Grindstaff et al. 2003; Boulinier and Staszewski 2008; Hasselquist and Nilsson 2009). Albumen contains maternally-derived lysozyme, an enzyme that destroys bacterial cell walls and is thus a major component of the antibacterial immunity of the egg (e.g. Trziszka 1994; Saino et al. 2002b). Avian eggs also contain substantial amounts of hormones, such as androgens (testosterone, androstenedione and dihydrotestosterone), estrogens and corticosterone (reviewed in Groothuis et al. 2005b; Gil 2008).

1.2. Hormone-mediated maternal effects

Hormone-mediated maternal effects are important as they have been shown to have a considerable impact on offspring development, phenotype and life-history traits in many vertebrate species (e.g. Hews et al. 1994; Clark and Galef 1995; Lovern and Wade 2001; Ryan and Vandenbergh 2002; Dufty et al. 2002; Uller and Olsson 2003; Groothuis et al. 2005b; Gil 2008). For example, in rodents, early exposure to hormones affects vital life-history traits such as breeding success, sexual behaviour and sex-ratio (reviewed in Clark and Galef 1995; Ryan and Vandenbergh 2002). In reptiles, hormone exposure has been shown to be related to the expression of male morphs (Hews et al. 1994), but also growth and immune function (Uller and Olsson 2003; Uller et al. 2007). These long-lasting effects of early exposure to androgens may arise from androgens modifying the endocrine system of the offspring, either through modification of hormone production or through organizational effects that, for example, change hormone receptor sensitivity in target tissues (Carere and Balthazart 2007; Groothuis and Schwabl 2008). Most importantly, early hormone exposure can have multiple effects (both positive and negative and also sex-specific) on the phenotype in different time-scales on an animal's life-span, and may therefore function as a proximate means for central life history trade-offs in phenotypic development and reproductive strategies (Ketterson and Nolan 1992, 1999; McGothlin and Ketterson 2008; Groothuis and Schwabl 2008). The fact that early hormone exposure has been shown to affect phenotypes also in the long-term makes it possible to study its role in the context of evolutionary processes.

1.3. Effects of yolk androgens on offspring phenotype

The study of avian yolk androgens began in 1993 when they were discovered by Hubert Schwabl (Schwabl 1993), and there has been continuously increasing interest in this topic ever since. Early androgen exposure (i.e. yolk androgens) can affect various physiological, morphological and behavioural traits of the offspring both in the short and long term (reviewed in Groothuis et al. 2005b; Gil 2008). There is a large number of studies reporting the effects of yolk androgens on the early stage of life: positive effects on growth, early competitiveness, begging intensity and survival of the nestlings have been mainly reported (e.g. Schwabl 1996; Eising et al. 2001; Eising and Groothuis 2003; Pilz et al. 2004; Groothuis et al. 2005a; Tschirren et al. 2005; Navara et al. 2006; Müller et al. 2007a). However, effects on offspring are not always beneficial as yolk androgens have been found, for example, to suppress immune system (e.g. Groothuis et al. 2005a; Müller et al. 2005; Navara et al. 2005; Sandell et al. 2009). An originally beneficial trait (such as high growth rate) could also lead to detrimental effects later in life (Metcalfe and Monaghan 2001; Marshall and Uller 2007). Recently, there has been an increasing number of studies reporting long-lasting effects of yolk androgens on, for example, adult plumage characteristics and behaviour (Strasser and Schwabl 2004; Daisley et al. 2005; Eising et al. 2006; Rubolini et al. 2006; Tschirren et al. 2007; Müller et al. 2008; Müller and Eens 2009). Effects of yolk androgens have often also been found to be sex-specific (e.g. Groothuis et al. 2005b; von Engelhardt et al. 2006; Saino et al. 2006; Rutkowska and Cichoń 2006; Rutkowska et al. 2007; Pitala et al. 2009). However, only relatively few studies have looked into the long-term effects of yolk hormones on fitness-related traits (Rubolini et al. 2007; Rutkowska et al. 2007; Tschirren et al. 2007; Partecke and Schwabl 2008; Müller et al. 2009b), and most of these effects have only been demonstrated in domestic or captive species. In those systems, it is difficult to study whether the effects of yolk androgens are adaptive.

1.4. Variation in yolk androgens – causes and potential adaptive value

Substantial variation in yolk androgen levels both within and between clutches have been found in all avian species studied (reviewed in Groothuis et al. 2005b; Gil 2008). Yolk hormone levels have in many cases been found to either increase or decrease with laying order within the clutch (e.g. Schwabl 1993; Gil et al. 1999; Lipar et al. 1999; Royle et al. 2001; Reed and Vleck 2001; Groothuis and Schwabl 2002; Pilz et al. 2003; Gasparini et al. 2007). In the light of the positive effects of yolk androgens on offspring growth and competitive abilities (see above), within-clutch variation has been interpreted as a means to adjust brood size adaptively depending on environmental conditions: High androgen levels in the last eggs may compensate for the competitive disadvantage of the late-hatching offspring (Schwabl 1993; Schwabl et al. 1997; Lipar et al. 1999) or alternatively, low androgen levels in the last eggs may facilitate brood reduction (Schwabl et al. 1997). An alternative hypothesis (the offspring diversity hypothesis) proposes that parents are selected to produce phenotypically variable offspring, which might be a risk-spreading strategy in variable environments (Laaksonen 2004).

The magnitude of variation among clutches exceeds the within-clutch variation substantially in most species studied (Groothuis et al. 2005b). Among-clutch variation in yolk androgen levels has been associated, for example, with breeding density (Schwabl 1997; Reed and Vleck 2001; Mazuc et al. 2003; Pilz and Smith 2004), mate attractiveness or age (Gil et al. 1999; Gil et al. 2004; Michl et al. 2005; Gil et al. 2006), parasite load (Tschirren et al. 2004), food supply and female condition (Verboven et al. 2003; Gasparini et al. 2007; Tobler et al. 2007a). However, the adaptiveness of among-clutch variation is not well known, as experiments testing the adaptive significance, especially including sex-specific effects, have very rarely, if never, been conducted (but see e.g. Müller et al. 2007a; Tschirren et al. 2005, 2007; Müller et al. 2009a). In particular, long-term effects on adult survival and reproductive success in the wild have been very rarely investigated. Furthermore, the proximate physiological mechanisms affecting accumulation of yolk androgens in eggs is still under debate (passive transfer of androgens to eggs or any degree of regulation), and the nature of this mechanism may limit the adaptive potential of yolk androgens (Groothuis and Schwabl 2008). Also the generality of the allocation patterns within species is poorly known, as studies are often conducted in only one study population (but see e.g. Hahn et al. 2005; Badyaev et al. 2006b).

Understanding the evolution of yolk androgen levels and their potential adaptive value requires considering its costs and benefits in terms of future survival and reproductive success of not only the offspring, but also the parents (e.g. Marshall and Uller 2007; Müller et al 2007b). Large fitness costs of high yolk androgen levels for the parents could select allocating levels that are suboptimal for the offspring, leading to parent-offspring conflict (e.g. Trivers 1974; Godfray 1995; Müller et al. 2007b). This conflict could select for offspring to modify their response to androgens in a way that is adaptive for themselves (e.g. via altered receptor sensitivity, Müller et al. 2007b; see also Paitz et al. 2010), and lead to a potential evolutionary “arms-race”. Direct costs for females could occur if depositing high hormone levels in the egg implies that the mother is exposed to high circulating hormone levels herself (Groothuis and Schwabl 2008), causing, for example, delayed reproduction or reduced clutch size (e.g. Veiga et al. 2004; Clotfelter et al. 2004; Rutkowska et al. 2005). Indirect costs for both parents could occur if rearing a brood from eggs with high androgen levels is very energy demanding (for example due to high metabolic rate or activity and thus high daily energy expenditure of the chicks, Tobler et al. 2007b), reducing survival or breeding success of the parents in the following breeding season. To my knowledge, the potential long-term costs of yolk androgens on parents have not yet been investigated.

Moreover, sexual conflict between the parents over parental investment may affect allocation decisions and shape the evolution of yolk hormone deposition (Groothuis et al. 2005b; Houston et al. 2005; Michl et al. 2005; Lessells 2006; Moreno-Rueda 2007; Müller et al. 2007b; Tschirren and Richner 2008; Müller et al. 2010); In species with bi-parental care, females and males are in conflict over the amount of parental care they provide to their offspring (Trivers 1972; Arnquist and Rowe 2005; Houston et al. 2005; Lessells 2006; Hartley and Royle 2007) since increased investment reduces the future reproductive success and survival prospects of each parent (Williams 1966; Roff 1992; Parker et al. 2002). Therefore it would be favourable for one parent to shift labour to the other (Parker et al. 2002; Houston et al. 2005), for example by changing either offspring traits or his or her own behavior to which the partner responds (Westneat and Sargent 1996; Arnquist and Rowe 2005; Lessells 2006). As offspring phenotype, for example competitive ability of chicks, chick begging and growth (see references above), is affected by yolk androgens, these hormones may offer a mechanism through which female birds could influence offspring traits in ways that increase the relative investment by the male. This hypothesis has been proposed several times in the recent literature, but as far as I know only few studies have formally tested it (Tschirren and Richner 2008; Müller et al. 2010, I).

1.5. Digit ratio – a potential phenotypic indicator for early hormone exposure

Studying fitness consequences of early androgen exposure in the wild is essential for understanding its adaptiveness and evolutionary potential. However, in practise, measuring early hormone environment and the phenotype or success of the same individual is challenging in most vertebrates. Thus, an easily measurable phenotypic indicator of early hormone exposure would be of great value. Digit length ratios have been proposed for such an indicator, as they seem to reflect hormone exposure during early development, which is also important for the development of other traits (pleiotropic effects of hormones) (Clark and Galef 1995; Manning et al. 1998; Manning 2002; Dufty et al. 2002; Cohen-Bendahan et al. 2005; Groothuis et al. 2005b; McIntyre 2006; Garamszegi et al. 2007; Hönekopp et al. 2007). Digit ratios have indeed been shown to correlate with several phenotypic traits and behaviors (Manning et al. 1998; Manning 2002; McIntyre 2006; but see Putz et al. 2004 for a critical review). Few studies have investigated this topic in egg-laying animals, but the existing two experimental studies indicate that digit ratios may reflect the maternally-derived early hormone environment (Romano et al. 2005; Saino et al. 2007b).

1.6. Geographic variation in maternal effects

Previous chapters have mostly considered variation in yolk androgens and other egg components in a within-population context. However, when populations inhabit different selective environments, and there is spatial variation in the adaptive benefits of maternal resources, among-population variation in the allocation of maternal effects could be selected for, potentially creating local adaptations. Consequently, maternal effects could contribute to population divergence and among-population differences in life-history traits (adaptive ecological divergence, e.g. Mayr 1947; Badyaev et al. 2002; Funk et al. 2006; Räsänen and Kruuk 2007). The potential for maternal effects to play a role in evolutionary change and creating population differentiation (via changing response to selection) has been acknowledged by theoreticians earlier (Kirkpatrick and Lande 1989; Wade 1998; Wolf et al. 1998; see also Badyaev and Uller 2009), but has only recently gained empirical interest (Reinhold 2002; Badyaev et al. 2002, 2003; Grindstaff et al. 2003; Badyeav et al. 2006b; Räsänen and Kruuk 2007; Saino et al. 2007a; Badyaev 2008; Uller 2008; Badyaev and Uller 2009; Biard et al. 2009). The prerequisites for maternal effects in eggs mediating divergence in offspring traits among populations (via natural selection) include: 1) allocation of egg components should affect fitness; 2) allocation of egg components should be heritable; and 3) there should be high spatial variation in the adaptive benefits of maternal effects, but low temporal variation (see e.g. Kawecki and Ebert 2004). Deposition of resources in eggs seems to fulfil these prerequisites, as allocation in eggs has been shown to affect offspring fitness (Bernardo 1996b; Mousseau and Fox 1998a, b; Groothuis et al. 2005b; Hasselquist and Nilsson 2009), and often includes a genetic component (Christians 2002; Hartmann et al. 2003; Grindstaff et al. 2003; Zhang et al. 2005; Cucco et al. 2006; Sellier et al. 2007; Boulinier and Staszewski 2008; Tschirren et al. 2009). For example, differential maternal allocation may create sexual size dimorphism inducing population divergence (Badyaev et al. 2002, 2003). However, the first step in understanding the potential role of egg components in population differentiation would require quantifying the intra-specific variation in egg components among populations. To my knowledge this has been done for very few maternal effects in any animal on a large geographical scale (with the exception of variation in egg size in vertebrates and invertebrates: Iverson et al. 1993; Rowe 1994; Hõrak et al. 1995; Azevedo et al. 1997; Johnston and Leggett 2002; Encabo et al. 2002; Räsänen et al. 2005).

1.7. Aims of the thesis

In this thesis I addressed several novel and unanswered questions related to the ecological and evolutionary significance of yolk androgens and other maternal effects in avian eggs, using the collared flycatcher (*Ficedula albicollis*) and the pied flycatcher (*Ficedula hypoleuca*) as model species. To cast light on the evolutionary pressures on allocation of yolk androgens, I studied the benefits, potential costs and constrains of yolk androgens from the perspective of all family members (mother, father and offspring of both sex). I first studied the potential role of yolk androgens in sexual conflict over parental care, i.e. whether yolk androgens may offer a mechanism through which females could influence offspring traits in ways that increase the relative investment by the male (paper I). This was done by using both correlative data on yolk androgen levels and experimental manipulation of yolk androgens in a wild population of collared flycatchers. I further studied the potential long-term indirect costs of raising a brood with high yolk androgen level on parent generation (II) as fitness costs for the females may constrain the adaptive allocation (from the offspring perspective) of yolk androgens.

Demonstrating fitness-effects of early hormone exposure for the offspring is essential for studying the adaptiveness or evolutionary potential of these maternal effects. However, to my knowledge, only one such study exists from the wild from birds (Tschirren et al. 2007; see Uller et al. 2007 for reptiles). Thus, I tried to fill the gap in knowledge about the long-term effects of yolk androgens: I studied the effects of experimentally manipulated yolk androgen levels on recruitment and breeding success in a wild population of collared flycatchers (II) and the long-term effects of such treatment on various behavioural traits in pied flycatchers (III). I also investigated whether digit ratios can be used as an easy-to-measure phenotypic indicator for early androgen exposure (IV), potentially facilitating the laborious study of long-term fitness effects of yolk androgens.

Finally, I quantified the within- and among-population variation in yolk androgens and other important egg components on a large geographical scale to investigate whether allocation of egg components may reflect to a greater extent phenotypic plasticity or local adaptation due to varying selection pressures in different environments. The latter would indicate a potential role of maternal effects in population differentiation (V). I further studied the association of environmental conditions and individual quality on allocation of yolk androgens, as well as other egg components on a large spatial scale to see if any general allocation patterns existed (V).

2. METHODS

2.1. Study species

My study species were two closely related passerine bird species, the collared flycatcher *Ficedula albicollis* (I, II) and the pied flycatcher *F. Hypoleuca* (III, IV, V). Both species are small, insectivorous, cavity-nesting, migratory birds that winter in sub-Saharan Africa and breed in Europe during the summer (e.g. Lundberg and Alatalo 1992). The breeding range of the species is somewhat different: the pied flycatcher breeds in most of Europe, whereas the collared flycatcher breeds mainly in central and eastern Europe, with the exception of the Swedish islands Gotland and Öland. The reasons for selecting these species for model organisms are the following: 1) Both species can be attracted to breed in large numbers in nest boxes, and thus a large sample size can be acquired; 2) Both species tolerate disturbance at the nest well and thus manipulations, cross-fostering, catching of the adults and measurements of the offspring can be done without disturbing their breeding; 3) High natal philopatry in the collared flycatcher facilitates follow-up of the recruits. In the island population on Gotland, where this study was carried out, the recruitment rate of the chicks is as high as 10%; 4) Considerable background information (see below), including information on yolk androgen levels, exists as these species are among the most common avian models in ecological and evolutionary studies.

Components in the eggs of the two flycatcher species show considerable variation within and among clutches: Androgen levels seem to vary with laying order in the pied flycatcher (Gil et al. 2006; Tobler et al. 2007a), but not in the collared flycatcher (in a Hungarian population, Michl et al. 2005). Yolk testosterone and androstenedione allocation has been shown to be repeatable within females (Tobler et al. 2007a; Tschirren et al. 2009) and yolk testosterone allocation has been found to be heritable (Tschirren et al. 2009). Among-clutch variation in yolk androgen levels is associated with environmental factors such as timing of breeding (Michl et al. 2005; Tobler et al. 2007a), food supply (Laaksonen, T. unpublished), social stimulation (Hargitai et al. 2009), female characteristics (condition, Tobler et al. 2007a; Tschirren et al. 2009; but see Michl et al. 2005), and male quality (age, Michl et al. 2005; Laaksonen, T. unpublished). Considering the other egg components, yolk carotenoid and immunoglobulin levels have been found to vary in relation with laying order (Hargitai et al. 2006a; Török et al. 2007), timing of breeding (Hargitai et al. 2009), female condition (Hargitai et al. 2006a, 2009) and caterpillar availability (carotenoids: Hargitai et a. 2006b; Török et al. 2007). For albumen lysozyme levels, no information in the study species exists. Furthermore, the sex-specific levels of yolk androgens or any other egg components are not known for these two species. The observed variation within and among clutches in the two flycatcher species makes them interesting models for studying the consequences and potential adaptive value of this variation.

2.2. Study areas

For the collared flycatchers, the experiments were carried out in a relatively isolated population on the island of Gotland, Sweden (Fig.1; papers I and II). This nest box population was founded as early as 1980 by Lars Gustafsson. It consists of several wood plots with ca. 1600 nest boxes. Most of the plots are deciduous woodlands dominated by oak, *Quercus robur L.* and ash, *Fraxinus excelsior L.*, with a dense understorey of hazel, *Corylus avellana L.*, and hawthorn, *Crataegus spp*. (e.g. Pärt and Gustafsson 1989). Pied flycatchers were studied in a nest box population in Turku, Finland (established on a larger scale in 2004) (papers III and IV). In this population, 230 nest boxes are available in habitat patches dominated by oak (and in some places birch, *Betula pendula* and pine, *Pinus sylvestris*). Data on egg components was also collected from several pied flycatcher populations across Europe from which data on life-history traits was available (Fig 1., paper V).

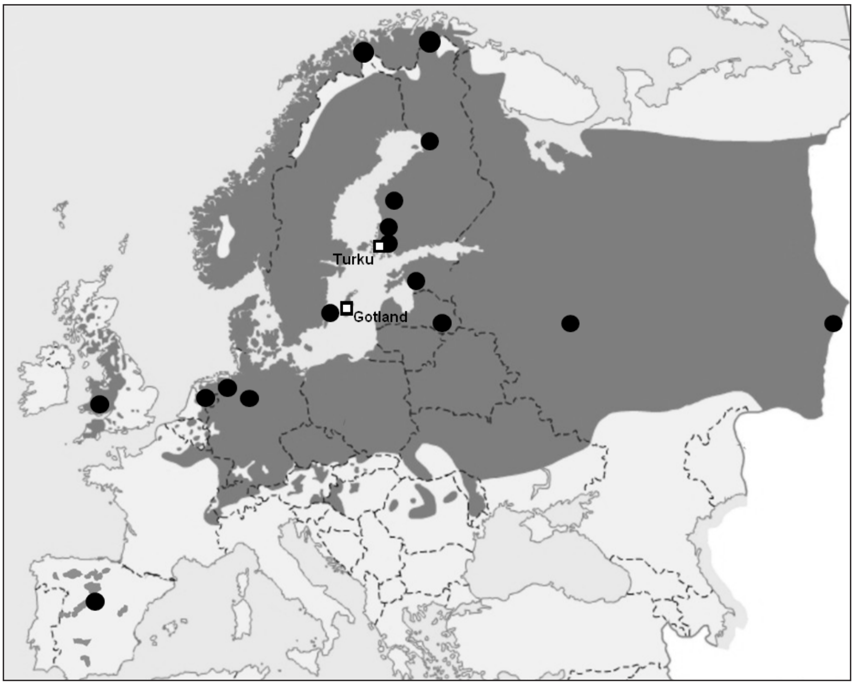

Fig.1. Locations of the main study populations (white squares): population of collared flycatchers in Gotland, Sweden (I, II) and pied flycatchers in Turku, Finland (III, IV, V). Black dots indicate the study populations included in the study of geographical variation in egg components (V). Grey area indicates breeding range of pied flycatchers in Europe (map modified from: Birds of the Western Palearctic, version 2.0.1., Oxford University press, 2003).

2.3. General methods

2.3.1. Yolk androgen manipulation

The same method was used in yolk androgen manipulations for collared flycatchers in Gotland in the spring of 2007 (I, II) and for pied flycatchers in Turku in the spring of 2008 (III, IV). Nest boxes were regularly checked every third day to determine laying dates. Both flycatchers lay one egg per day, and on the estimated day of clutch completion (i.e. day of laying of the 6th egg), each clutch was randomly assigned to either androgen treatment or control treatment. In the androgen treatment, yolk androgen levels (testosterone and androstenedione) were elevated by injecting the yolk with androgen solution. In the control treatment, only oil was injected. All the eggs in a clutch were treated the same way. The amount of injected androgens was calculated using previous data on natural yolk androgen levels in these populations and it corresponded to the difference between mean and maximum values of androgens per yolk, ensuring that final yolk androgen level in the androgen-treated clutches was, on average, at the upper limit of the natural range.

2.3.2. Egg collection and analyses of yolk androgens and other egg components

Eggs were collected for androgen analyses in papers I, IV, and V. Nest boxes were regularly checked every third day to determine the progress of nest building and egg laying. When one or two eggs were found in the nest they were marked, and the nest box was visited in the following day(s) to collect the third (or in some cases the fourth) egg of the clutch on the day of laying. The collected egg was replaced by a dummy egg in each nest. Eggs were frozen until analyses. The average clutch size in these species is six eggs, so the third egg represents one of the middle eggs of a clutch. The egg components may vary systematically with laying order (e.g. Ojanen 1983; Saino et al. 2002a; Groothuis et al. 2005b; Tobler et al. 2007a; Gil 2008), but the middle eggs should represent the average values of the clutch. Androgens were analysed with either radioimmunoassay (RIA) (I) or enzyme immunoassay (EIA) (IV, V). In the study on among-population variation in egg composition, other egg components (egg mass, yolk mass, yolk immunoglobulins, yolk total carotenoids and albumen lysozyme enzyme) were also analysed (see paper V for details).

2.3.3. Breeding data

In both species and all populations, collection of the breeding data followed standard protocols. Data was collected for collared flycatchers in 2007-2009 (I, II) and for pied flycatchers in 2005 (IV), 2007 (V) and 2008 (III, IV). Briefly, nest boxes were regularly checked every second or third day to determine the progress of nest building and egg laying. The laying date of the first egg was recorded, and nests were monitored throughout the breeding season to record final clutch size, hatching date, number of hatchlings and fledglings. Parent birds were caught during the chick feeding period (days 6-10 after hatching) using a swing-door-trap, measured (wing length, tarsus length and body mass) and aged as 1-year old or older. In pied flycatchers, male plumage colour morph was measured as a Drost score ranging from I (fully black dorsal coloration) to VII (fully brown dorsal coloration) (Drost 1936). Chicks were measured (tarsus length and body mass) 12 days after hatching. Nestlings were blood sampled for sexing in some of the collared flycatcher nests in 2007 (Pitala et al. 2009) and in all pied flycatcher nests in 2005 (IV) and 2008 (III, IV).

2.4. Study questions and specific methods

2.4.1. Yolk androgen manipulation experiment in collared flycatchers

Yolk androgen levels were experimentally manipulated in a large sample of collared flycatcher nests (120 androgen-treated and 120 control clutches, see methods above) in 2007 to address several questions. Firstly, the potential role of yolk androgens in sexual conflict over parental care was studied in half of these nests (I, II). To estimate the relative work load of the parents, the feeding rates of parents at the manipulated nests were measured (9 days post-hatching) by using video recorder. If the feeding rates of males would be affected by the androgen treatment, this could imply that males respond more to traits affected by yolk hormones, potentially facilitating manipulation by females. In another set of nests during the same breeding season, I collected one egg per clutch to estimate the average yolk androgen levels and cross-fostered the chicks (these nests were not included in the androgen-manipulated or control nests). I did this to examine whether there is a correlation between male proportion of feedings and yolk androgen level, facilitating the role of yolk androgens in sexual conflict over parental care. Secondly, in one half of the androgen-manipulated and control nests the short-term effects of yolk androgen manipulation on nestling growth and immune capacity were investigated. These results have been published elsewhere (Pitala et al. 2009) and they do not constitute a part of this thesis work. I will, however, refer to these results when they add to the understanding of my results. Thirdly, I studied the long-term effects of raising a brood with high androgen levels on the parent's return rate and breeding success during the following breeding season (in 2008) (II). If any costs to males were found, this might indicate the potential role of yolk androgens in sexual conflict over parental care. However, if long-term costs to females occur, this might constrain the optimal allocation of yolk androgens from the offspring perspective, potentially leading to a parent-offspring conflict. Finally, by monitoring the recruitment and breeding success of the offspring from androgen-treated and control nests during the two following breeding seasons (2008 and 2009), I studied whether there are long-term fitness effects of high yolk androgen levels for the offspring (II).

2.4.2. Yolk androgen manipulation experiment in pied flycatchers

I studied the effects of yolk androgens on the behavioural traits of pied flycatchers by manipulating yolk androgen level, raising the birds in a common-garden environment in captivity and measuring behaviour with standard tests (III). Briefly, androgen levels of the eggs were manipulated (see protocol above) and 12 days post-hatching two chicks, a male and a female, from 30 broods (15 androgen-treated and 15 controls) were transferred to captivity. Ten new foster broods with a brood size of six chicks were arranged from these 60 nestlings. Two foster parents fed each brood in the aviaries until it was certain that the young could eat independently (at the age of ca. 40 days), after which the foster parents were released to the wild again. Each brood was kept in an aviary size 2.5 m × 3 m × 2.5 m (at the Botanical garden of University of Turku), and after releasing the parents, birds were kept in groups of 12 (aviary size 5 m × 3 m × 5 m) over the winter. Temperature and photo period were adjusted to mimic the conditions birds would normally meet during migration and in wintering areas. Birds were fed with diverse live food *ad libitum*.

The birds were involved in several behavioural experiments: 1) The *novel environment experiment* was conducted to measure the exploration and activity of the individuals in a novel environment, an observation room of the size 4 m × 2 m × 2.5 m, which contained five artificial wooden trees. 2) The *novel object experiment* was conducted to measure the boldness of individuals towards novel objects (i.e. neophobia) and their activity in the presence of one. In this experiment, the novel object, a toy rubber duck, was placed on a perch in a bird's home cage. The latency to approach the object and activity of the bird were measured. The experiments were conducted when the birds were ca 12 weeks old and repeated when they were ca 29 weeks old. The experimental protocol of the two above-mentioned experiments was modified from Verbeek et al. (1994). 3) The *dominance experiment* measured the dominance/aggressiveness of the individuals over food and their activity in a social situation. The experimental protocol was modified from Strasser and Schwabl (2004). Briefly, one day before testing, one androgen-treated and one control treated individual (of same sex and approximately same body mass) were placed in a cage divided in two with a net. After an overnight fast, the net partition was removed and a feeder allowing only one bird to feed at a time was placed in the centre of the cage. I determined the dominance rank based on the behaviour of the birds (e.g. latency of each bird to arrive at the feeder, monopolising the perches, forcing the other one to sit on the floor of the cage), and recorded the activity level in this social context. 4) The *Anti-predator behaviour experiment* measured variation in anti-predator behaviour and risk-taking (i.e. boldness) after a simulated predator attack. The experimental protocol was modified from Quinn and Cresswell (2005). The test bird was placed in a test cage situated on the floor in the corner of the experimental room. After a habituation period, a stuffed sparrow hawk (*Accipiter nisus*) in an attacking glide posture was released remotely and slid down a line over the test cage. The response of the bird to the appearance of the hawk was classified as an *escape response* when the bird tried to fly away, or *freezing* when the bird remained almost or entirely motionless, or tracked the flight path of the sparrow hawk until it went out of view. After the predator had disappeared from sight, the time the birds took to resume activity was recorded. The dominance and anti-predator experiments were conducted when the birds were ca. 15 weeks old.

2.4.3. Yolk androgens and digit ratios

I investigated whether digit ratios can be used as an easy-to-measure phenotypic indicator for early androgen exposure (IV). I studied the effects of experimental elevation of yolk androgen level on digit ratios in the captive pied flycatchers (see above) when the birds had fully grown and reached independence. Furthermore, in another set of nests (in 2005) correlations between naturally occurring yolk androgen levels and digit ratios of the nestlings were studied. This was done by collecting one egg (the middle egg, see methods as above) from a clutch to estimate the average androgen level in a brood and measuring digit ratios from 12 day-old nestlings. The digit ratios of the parents were also measured, and potential sex differences in nestlings, parents and captive individuals were examined. The digit ratios of adults and nestlings were measured by straightening the toes and pressing them against a glass sheet (with a millimeter grid for calibration on the side), which was attached to a digital camera. Photos were examined with a graphics program (ImageJ), and the lengths of the 2nd, 3rd and 4th toes were measured and their ratios calculated.

2.4.4. Geographical variation in yolk androgens and other egg components

I quantified within- and among-population variation in yolk androgens and other important egg components on a large geographical scale to explore whether allocation of egg components may reflect either local adaptation due to varying selection pressures in different environments (populations) or phenotypic plasticity (V, Fig 1.). The former would indicate a potential role of maternal effects in population differentiation. To do this, progress of breeding was monitored in several pied flycatcher populations across Europe to record laying dates. One egg per nest (the middle egg) was collected and other essential breeding parameters were recorded (see above). Several important egg components (egg and yolk mass, yolk androgens, yolk immunoglobulins, yolk total carotenoids and albumen lysozyme enzyme activity) from the collected eggs were measured using standard methods in one laboratory. I also compiled long-term life-history data and data from environmental variables from the study populations for a twofold purpose: I quantified spatial and temporal variation in several important indicators of environmental variation to study whether the different populations might represent different selective environments. I further studied the association of environmental conditions (geographic location, temperature, habitat, relative quality of the breeding season (relative to an average year) and individual quality (indirectly via laying date and clutch size)) on allocation of yolk androgens and other egg components on this large spatial scale. The results would reveal whether any general allocation patterns existed.

3. MAIN RESULTS AND DISCUSSION

3.1. Short- and long-term effects of yolk androgens on parent generation (I & II)

Yolk androgens did not impose any short- or long-term effects on female or male parents (I, II): Male feeding rates or male's proportion of total feedings did not differ between control and androgen-manipulated nests, suggesting that males do not adjust their feeding differentially to high yolk androgen levels in the collared flycatcher (I). In line with the lack of short-term effects, raising a brood from eggs with high yolk androgen levels did not induce long-term fitness costs for the parents: neither return rate, nor breeding success in the following season differed between parents of androgen-treated and control nests (II). The lack of sex-specific short- and long-term effects suggests that females and males are not responding differently to signals of nestlings from eggs with high and low yolk androgen levels, and thus yolk androgens *per se* do not appear to mediate sexual conflict over parental investment in this species. The two other existing experimental studies also failed to show that males respond more to offspring from eggs with high yolk androgen levels (Tschirren and Richner 2008; Müller et al. 2010). The lack of male response to manipulation may indicate that males have evolved responses to specific offspring signals that cannot be manipulated by females and/or ignore signals that can be manipulated by females (Müller et al. 2007b). I nevertheless found that the male's proportion of total feeding visits increased with increasing androgen levels in the foster eggs, which suggests that females might be able to manipulate males via other maternal effects correlated with yolk androgens (I).

3.2. Effects of yolk androgens on offspring generation (II & III)

High yolk androgen levels had both short-term and long-term effects on morphology, behaviour and fitness-related traits in the two flycatcher species (II and III, see also Pitala et al. 2009). Interestingly, most of the effects of high yolk androgen levels were sex-specific: only males were affected. Elevated yolk androgen levels resulted in a higher probability of exploring the novel environment and higher activity in the novel object experiment in male, but not in female captive pied flycatchers (III). In response to a simulated predator attack, males from the androgen treatment mainly showed freezing behaviour whereas males from the control group showed escape behaviour (III). These behavioural effects of yolk androgens may have fitness consequences in the wild, as increased activity or exploration may lead to improved foraging ability (e.g. Verbeek et al. 1999; Marchetti and Drent 2000; Dingemanse and de Goede 2004). On the other hand, high activity/mobility may also increase predation risk (e.g. Réale and Festa-Bianchet 2003; Sih et al. 2003; Dingemanse et al. 2004; Sih et al. 2004; Boon et al. 2008; Smith and Blumstein 2008). Also, performing proper anti-predatory behavior has direct effects on survival.

I found that high early androgen exposure lowered the local recruitment rate of male, but not female offspring in a wild population of collared flycatchers (II). There may be several alternative explanations for this result, since it might reflect lower survival but also non-breeding/failure to breed (as capturing males during the nestling phase excludes failed nests), or longer dispersal distance. Support for the high mortality alternative comes from the effects of yolk androgens on early development, as males from the androgen treatment were smaller in body size than controls (12d post-hatching, Pitala et al. 2009). Offspring size and condition are known to strongly affect post-fledgling survival in many species, including collared flycatchers (Gustafsson and Sutherland 1988; Lindén et al. 1992; Potti et al. 2002). Alternatively, the effects of yolk androgens on male behavior (III) could lead to increased predation risk (see references above). Higher mortality may also be caused by high androgen levels having a suppressive effect on the immune system (reviewed in Ketterson and Nolan 1992, 1999; Groothuis et al. 2005b; Gil 2008), potentially in a sex-specific way (Rutkowska et al. 2007). The low recruitment of males from androgen-elevated eggs need not necessarily be an effect of lower survival. The effects of androgens on male behaviour or phenotype (for example on plumage characteristics, aggressiveness or song quality, reviewed in Groothuis et al. 2005b; Gil 2008; III) may affect male attractiveness or their ability to occupy high quality territories, leading to low recruitment. This may also cause nest failure, for example via androgens decreasing male parental care (Ketterson and Nolan 1992, 1999). High yolk androgen levels may also lead to longer natal dispersal distances (dispersing outside the study area) (Tschirren et al. 2007), causing low male capture rate in the natal population (see Doligez et al. 2009). However, even if my results could be explained by dispersal, androgen-treated males may still face a fitness cost, as dispersing individuals often have lower fitness than philopatric individuals (reviewed in e.g. Doligez and Pärt 2008).

I found no effects of yolk androgen manipulation on breeding success (age at first breeding, timing of breeding, clutch size, hatching success and number of fledglings) of the female or male recruit collared flycatchers. Thus, the lower recruitment of androgen-treated males (II) very likely reflects a fitness cost, as the low recruitment was not compensated for by higher breeding performance. Furthermore, the breeding success of androgen-treated and control individuals was not differently affected by the environmental conditions during the breeding season (II). Maternal effects are assumed to provide a mechanism translating variation in environmental conditions into adaptive phenotypic variation of offspring (Mousseau and Fox 1998a, b), but as experiments testing the adaptive significance of yolk androgens have rarely been conducted (see e.g. Badyeav et al. 2006c; Müller et al. 2007a; Tschirren et al. 2007; Müller et al. 2009a, paper II and Love and Williams 2008 for corticosterone), it is not yet clear in which environmental or social conditions high yolk androgen allocation is adaptive in the long run.

Why did elevation of yolk androgen levels only affect males? Unfortunately, the proximate mechanisms of the sex-specific effects of yolk androgens are not well known. It is possible that the organizational effects of yolk androgens differ between the sexes due to differences in androgen sensitivity, possibly mediated by differences in the timing or location of androgen-receptor expression, or in the aromatization of androgens (as suggested by Sockman et al. 2008). Furthermore, if there are sex-specific differences in yolk androgen levels or in androgen production by the embryos (e.g. reviewed by Groothuis et al. 2005b), with male eggs possibly containing higher androgen levels, increasing already high androgen levels may lead to more pronounced effects in males than in females. Unfortunately, for my study species the sex-specific hormone levels are not known.

3.3. Evolution of hormone-mediated maternal effects – costs, constrains and family conflicts (I, II & III)

For a full understanding of the evolution of hormone-mediated maternal effects, it is essential to study fitness consequences and constraints from the perspective of all family members, as conflicts and trade-offs between male and female parents (sexual conflict), parents and offspring (parent-offspring conflict) and male and female offspring (sex-specific antagonistic effects) may shape the allocation of yolk androgens. My results indicated that yolk androgens do not appear to be involved in sexual conflict over parental investment (see above). Conflict between parents and offspring over parental investment may further shape the evolution of yolk androgen allocation (Trivers 1974; Godfray 1995; Müller et al. 2007b), as differential allocation of yolk androgens and other egg components does not always increase offspring fitness, but may do so for maternal fitness (Marshall and Uller 2007; Müller et al. 2007b). However, given the potential direct costs for females of allocating high levels of androgens (reviewed in Groothuis et al. 2005b) and the fitness costs of high yolk androgen levels for male offspring (II), my results might suggest that low levels of androgens would be beneficial for both the offspring and parent generation, implying lack of parent-offspring conflict. This further suggests that the original yolk androgen levels in experimental clutches may have been optimal.

The sex-specific antagonistic effects of yolk androgens complicate the evolution of yolk androgen allocation (and parent-offspring conflict): The fitness cost for males (II) should select for low levels of yolk androgens, even if high androgen levels have positive short-term effects in either sex or sex-specific antagonistic long-term effects. A way for mothers to overcome this constraint may lie in sex allocation: yolk androgens may be allocated differentially according to offspring sex (e.g. Müller et al. 2002; Gilbert et al 2005; Groothuis et al. 2005b; Badyaev et al. 2006a, b; Gil 2008), for example via sex-specific temporal clustering of the growing oocytes (e.g. Young and Badyaev 2004; Badyaev et al. 2005, 2006a, b). Alternatively, androgens may play a role in determining offspring sex (since the meiotic division occurs after yolk deposition; e.g. Rutkowska and Badyaev 2008; Uller and Badyaev 2009).

3.4. Yolk androgens and digit ratios (IV)

I did not find any association between digit ratios and maternally-derived yolk androgen level in the pied flycatcher, as experimental elevation of yolk androgen levels had no effect on digit ratios in adulthood, and there was no correlation between yolk androgen level measured from one egg in a clutch (a good proxy for the clutch mean) and the digit ratio of the remaining chicks at fledging (IV). This indicates that digit ratios may not be reliable markers of maternally-derived early androgen exposure. My results contrast with the findings of the few previous experimental studies in birds in which high androgen exposure in the yolk led to higher 2D:3D (but not 2D:4D or 3D:4D) in female pheasants (Romano et al. 2005) and elevation of estradiol levels in egg yolks decreased 2D:4D in males (Saino et al. 2007b). However, I only studied *maternally-derived* hormone levels, and it is highly possible that hormones of *embryonic* origin may have a greater role in digit development. In mammals it is widely presumed that mainly the steroids produced by the embryo, not by the mother, have pleiotropic effects on digit development and other traits (Manning et al. 2000; Manning 2002; McIntyre 2006). The relative contribution of different steroids (testosterone vs. estrogens) on digit ratio determination on different taxa is also unclear. A recent study on zebra finches suggests that estrogens are associated with development of digit ratios and explain the co-variation between digit ratios and other traits (Forstmeier et al. 2010). Furthermore, I found a sex-difference in 2D:3D and 3D:4D of adult birds, not yet apparent in fledglings or captivity-raised juveniles, which suggests that hormonal levels as late as during reproductive maturation may affect digit development (IV). Thus, the role of different steroids of maternal and embryonic origin as well as circulating hormone levels in adults on digit ratios in birds should be further examined.

3.5. Geographical variation in yolk androgens and other egg components (V)

When investigating variation in egg components of pied flycatchers on a large geographical scale, I found significant variation among populations in yolk androgens and other measured egg components (yolk mass, yolk immunoglobulins, yolk carotenoids and albumen lysozyme enzyme) with the exception of egg mass (V). However, despite the fact that I also found large among-population differences in indices of environmental variation (temperature, timing of breeding and clutch size), potentially acting as selective forces to induce local adaptations, population explained only a small share (ca. 10%) of the total variation for most egg components. The low among-population variation may indicate that there is no strong local adaptation in the allocation of resources to eggs, and that the potential for egg components to play a role in population divergence in offspring traits may not be strong, although it has been shown for some other maternal effects (e.g. Azevedo et al. 1997; Badyeav et al. 2002, 2003; Laugen et al. 2003; Räsänen and Kruuk 2007; Benton et al. 2008). Furthermore, for several egg components, variation was related to the relative quality of the breeding season, which suggests that allocation to eggs is to some extent a plastic response (adaptive or non-adaptive).

I further studied the association of environmental conditions and individual quality on the allocation of yolk androgens and other egg components across the breeding range to see whether any general allocation patterns exist. There was no common pattern explaining variation in different egg components; some were affected by common environmental factors such as habitat or temperature, whereas others were more affected by variation in individual quality or resources. The lack of such a common pattern is not surprising, as different egg components are subject to different constraints and selective forces influencing their deposition. Furthermore, the explanatory power of any of these environmental or individual factors was generally low (only a few percent, with the exception of carotenoids), suggesting that the patterns in each egg component are not strong.

4. CONCLUSIONS

In this thesis, I have shown that early androgen exposure has sex-specific, long-lasting effects on multiple fitness-related traits in the offspring (II, III). My findings emphasize that early conditions can have a major influence on an individual's life, and that the significant role of maternal effects on the development of the offspring phenotype should not be ignored (e.g. Bernardo 1996a, b; Rossiter 1996; Mousseau and Fox 1998a, b; Lindström 1999; Metcalfe and Monaghan 2001; Galloway 2005; Monaghan 2009).

My results indicate that yolk androgens may be associated with different life-history strategies via reconstructing offspring developmental niche and creating variation offspring phenotypes. However, the proximate physiological and molecular pathways behind the effects on yolk androgens on phenotype and behavior and particularly the sex-specific effects are very poorly known; they thus offer an interesting avenue for further studies.

These results also imply that yolk hormones may provide females with a means to manipulate offspring phenotype and even life-history strategies, potentially adapting their offspring to prevailing or anticipated conditions (Mousseau and Fox 1998a, b). However, the environmental or social conditions in which different phenotypes are favoured remained unresolved in this study (II) and should thus be further studied using a combination of hormonal manipulations and manipulation of the offspring environment. Using an easily measurable proxy for early androgen exposure would be compelling to facilitate long-term studies, but my findings emphasize that digit ratios should not be used as a phenotypic marker for maternally-derived early androgen exposure (IV).

Based on my results, the most potent evolutionary pressure shaping the allocation of androgens may lie in the direct costs of high yolk androgen levels for offspring (decreased male recruitment) (II), as no short- or long-term costs on the residual reproductive value of the mother were found (I, II), and variation in yolk androgen levels appeared not to be related to the role of androgens in sexual conflict between the parents (I, II). These results indicate that the trade-offs in the allocation of yolk androgens arise mainly through their effects on the fitness of male and female offspring, and not on parents. Thus, hormone allocation may be constrained by sexually antagonistic effects of hormones on females and males (e.g. Groothuis et al. 2005b), which may select for sex-specific allocation mechanisms (e.g. Rutkowska and Badyaev 2008; Uller and Badyaev 2009).

The fact that early hormone exposure was also shown to affect phenotypes and fitness-related traits in the long-term (II, III), together with the recent finding on the heritability of yolk androgen allocation (Tschirren et al. 2009), opens up the opportunity for hormone-mediated maternal effects to respond to selection and thus affect phenotypic change and evolutionary processes (e.g. Räsänen and Kruuk 2007; Uller 2008; Tschirren et al. 2009). For example, differential allocation of maternal effects may create sexual size dimorphism, inducing population divergence (Badyaev et al. 2002, 2003, 2006b). However, I found it unlikely that yolk androgens or other egg components would play a role in evolutionary change to create population differentiation and local adaptation (due to different selective environments) in the pied flycatcher, as within-population variation substantially exceeded among-population variation in a large-scale geographical comparison of early hormone levels and other egg components. Nevertheless, as my study was mainly explorative, more studies are needed to thoroughly understand the role of maternal effects in evolutionary change. Alternative pathways could facilitate local adaptation and evolutionary divergence: for example, populations may differ in within-clutch patterns of deposition or in relationships between egg components (e.g. Badyaev and Uller 2009). The patterns of variation among and within populations found in this study nevertheless provide a starting point for exploring the possible adaptive value of this variation, or lack thereof, for individual and population life-histories. To conclude, I think that for a full understanding of the evolution of hormone-mediated maternal effects, it is essential to study fitness consequences, physiological mechanisms and constraints from the perspective of all family members and in varying environmental and social contexts.

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Suvi Ruuskanen

Suvi Ruuskanen

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