Maternal hormones as a tool to adjust offspring phenotype in avian species

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Summary
Avian eggs contain substantial amounts of maternal hormones and so provide an excellent model to study hormone-mediated maternal effects. We review this new and rapidly evolving field, taking an ecological and evolutionary approach and focusing on effects and function of maternal androgens in offspring development. Manipulation of yolk levels of androgens within the physiological range indicates that maternal androgens affect behaviour, growth, morphology, immune function and survival of the offspring, in some cases even long after fledging. Descriptive and experimental studies show systematic variation in maternal androgen deposition both within and among clutches, as well as in relation to the sex of the embryo. We discuss the potential adaptive value of maternal androgen transfer at all these three levels. We conclude that maternal androgen deposition in avian eggs provides a flexible mechanism of non-genetic inheritance, by which the mother can favour some offspring over others, and adjust their developmental trajectories to prevailing environmental conditions, producing different phenotypes. However, the literature is less consistent than often assumed and at all three levels, the functional explanations need further experimental testing. The field would greatly benefit from an analysis of the underlying physiological mechanisms.

Keywords: Maternal effects; Androgens; Birds; Individual differentiation; Development; Phenotypic plasticity; Sex allocation; Hatching asynchrony; Sibling competition

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1. Introduction

Most studies of the effects of prenatal exposure to hormones deal with proximate mechanisms, using rodents as experimental animals, and are aimed at understanding normal and abnormal human development. The aim of this paper is to advocate the value of an ecological and evolutionary perspective, and to demonstrate the advantage of a comparative approach, using animal models other than rodents and field studies, in addition to studies under artificial laboratory conditions. We will focus on the potential of the mother to adjust adaptively the development of her offspring to prevailing environmental conditions by exposing the embryos to her hormones.

1.1. Phenotypic plasticity and maternal effects

Many organisms have the capacity to develop in a variety of ways [1,2]. These alternatives are referred to as ‘reaction norms’, ‘polyphenism’ or, in more general terms, ‘phenoplasticity’. In many cases, the developmental outcome is the result of a prediction about the conditions in which the new individual will subsequently live. An obvious route by which such a forecast is made is the parents. Parents have a powerful tool by which they can influence the development of their offspring, namely by influencing the environment in which the offspring develops. When the phenotype of the parents influences the phenotype of their offspring, these are called ‘parental effects’ or often, but less accurately so, ‘maternal effects’ [3]. A simple example is that of parental food provisioning during rearing, which influences the body mass of the young, which in turn may affect their ultimate reproductive success. Such a parental effect can come about in two ways (Fig. 1): first, there may be an indirect genetic effect so that food provisioning is dependent on the genome of the parents and the offspring may then inherit both the genes for optimal food provisioning as well as the non-genetic effects of being fed well, with potentially very interesting evolutionary consequences [4]. Second, there may be indirect environmental effects, as, for example, when the rate or quality of food provisioning is determined by the food availability in the environment. In this situation, the parents (as providers of food) translate or communicate environmental conditions to their young. Such non-genetic transmission of information, in contrast to genomic inheritance, is very flexible: parents can anticipate the conditions in which their young will live and thereby maximize the propagation of their own genes into the next generation.

Parental effects are much more widespread than previously thought and may in fact inflate heritability estimates. Furthermore, they can come about by a large diversity of pathways and at different times in the life cycle.
The aim of this paper is to highlight the possibility of adaptive parental effects, by the mother, that are induced during the prenatal phase. Such prenatal maternal effects are important for two reasons: firstly, it is well established that early environmental influences are more likely to lead to irreversible modifications. Secondly, the embryo, enclosed in the mother’s body, or in the egg produced by her, cannot easily register environmental conditions in order to adjust its development and is therefore reliant on information provided by the mother about these conditions.

1.2. The role of maternal hormones in modifying offspring development

Steroid hormones, such as gonadal hormones, are important mediators of prenatal maternal effects. Early exposure to these hormones can result in long-lasting organizing effects on brain and behaviour, a classical example of which is their involvement in sexual differentiation [5]. More recently, evidence is accumulating that these hormones also affect differentiation within the same sex (for a review, see Ref. [6]). Early exposure to androgens could mediate the development of different reproductive strategies in males, such as that of ‘territorial holders’ trying to monopolize females and ‘sneakers’ that try to steal copulations from the former (e.g. lizards, [7]). Another classical example of the organizing influence of gonadal hormones on the phenotype is the effect of intra-uterine position in rodents and pigs, which exposes embryos to the gonadal hormones produced by the neighbouring siblings in the same horn: depending on the sex of these siblings, the embryo is exposed to relatively high levels of either androgens or estrogens, affecting a whole suite of behavioural and physiological traits, leading to different phenotypes within each sex [8]. These examples may also involve some maternal influence on early hormone exposure. Eggs of reptiles contain substantial levels of maternal androgens. Female gerbils that were positioned in between two male siblings in utero produce male-biased litters, giving rise to relatively many similar positioned females themselves [8].

An example where maternal influence is more obvious comes from studies on the spotted hyena: high levels of circulating androgens in the female during pregnancy seem to cause the precocious development of the offspring and perhaps, to some degree, the female masculinization in this species too [9,10]. Finally, the influence of prenatal exposure to maternal glucocorticoids on later stress sensitivity and cognitive functions in rodents (e.g. Ref. [11] and several contributions to this issue) is another classical example of the long-lasting influences of early exposure to steroid hormones.

These examples also illustrate another reason for studying maternal effects mediated by steroid hormones: they have multiple effects on the phenotype and may therefore provide a proximate basis for a key concept in evolutionary ecology, that of trade-offs. A trade-off occurs when an organism is faced with a decrease in Darwinian fitness because a beneficial change in one trait (for example, a higher level of courtship behaviour to attract more females) is causally linked to a detrimental one in another (a decrease in parental care). Trade-offs (and hormones) represent linkages between traits that constrain the simultaneous evolution of two or more traits. The optimal outcome will be shaped by selection and may differ among individuals [12]. Because of their multiple effects, steroid hormones may underlie important trade-offs in many organisms, [13] providing a bridge between proximate and ultimate approaches in biology.

A third reason for studying steroid hormones as mediators of maternal effects is that their levels in the mother can strongly fluctuate as a function of the environment. It is well known that social factors, such as the frequency of aggressive challenges, stimulate androgen production (see below) while both social and non-social challenges strongly affect levels of glucocorticoids. Therefore, these hormones offer an excellent pathway to communicate the environment of the mother to her offspring.

1.3. Adaptation versus pathology

Because each individual is different, or lives in a slightly different environment, the optimal solution of the trade-offs is different for different individuals (individual optimisation). So, although phenotypes may differ in fitness, ecologists are of the opinion that variation around the mean of a certain trait could represent adaptive variation, unless it is selectively neutral (providing that the variation does not reflect inaccurate measurements). In contrast, scientists working in the field of animal physiology and medical sciences are more inclined to interpret deviation from the mean as potentially pathological. An example is the interpretation of the effects of early exposure to stress hormones mentioned above. Such effects are generally interpreted as detrimental, being an inevitable side effect of maternal stress. However, exposure to glucocorticoids may also have beneficial effects, [14] and in many cases the benefits may outweigh the costs. In rodents, prenatal exposure to stress or glucocorticoids has been shown to result in higher anxiety [15] and the consequence of this may be a reduction in risky behaviour. This has been demonstrated in a lizard species, in which prenatal exposure to these hormones resulted in offspring that were more risk-averse [16]. This may be adaptive in cases where maternal stress reflects an abundance of predators: by exposing her offspring to elevated levels of glucocorticoids, the mother produces more cautious offspring, thus having improved survival chances in an environment with many predators. In addition, it is known that enhanced prenatal exposure to glucocorticoids can lead to slower growth: since elevation of these hormones in the mother may also be induced by...
poor food conditions, inducing a slower rate of growth in her offspring is a maternal adjustment that may be adaptive for the offspring who encounters the poor environmental situation [17].

Likewise, prenatal exposure to enhanced levels of gonadal hormones may be favoured by natural selection. It has been suggested that effects of early exposure to gonadal hormones in rodents, inducing elevated levels of aggression and longer estrus cycles in females, may be important in the regulation of cycles of population density (for references, see Ref. [8]), although it should be noted that the results of two field studies were not entirely consistent with each other [18,19]. Similarly, there are several adaptive explanations for the high level of maternal androgens during pregnancy in the spotted hyena, mentioned earlier. One is that it induces female masculinization as an adaptation to the social system of the species. A clan often feeds together on the same prey and gaining food during this competitive situation is especially important for females, because they nurse their pups for a relatively long time and access to food correlates positively with their reproductive success. It is thought that because of prenatal exposure to maternal androgens the females, which even develop a pseudo-penis, become dominant over males to ensure this access to food [9]. This explanation has been challenged because blocking the action of early androgen exposure did only partially inhibit the development of the pseudo-penis, although the effect of the treatment on later aggression and dominance has not yet been established (for reviews, see Refs. [10,20]). The other explanation is that the high levels of maternal androgens induce, in the pup, precocious development of morphology and aggression. Since post-natal maternal investment in this species is very high, this has been interpreted as an adaptation to the need for siblicide under poor food conditions, to cull litter size to the available resources [10].

The ‘pathology-view’ may disregard how well natural selection results in the adaptation of organisms to their (fluctuating) environment. The ‘adaptation-view’ runs the risk of adaptive story telling with no hard data. Ideally, to assess whether maternal effects are adaptive, one should experimentally test the effects on the inclusive fitness of the mother. That is to say, how well her genes are propagated into subsequent generations. Ideally, such empirical verification should be conducted under the conditions in which these effects have evolved: the natural field situation. In practice, this is not easy to achieve. We can obtain adequate fitness estimates by measuring important aspects of it such as early growth, competitive behaviour and survival, but it is also important to note that different maternal effects may be highly correlated with each other. For example, maternal quality of nursing may be correlated with her body weight and both may independently influence offspring body weight. Therefore, experimental manipulation of one particular pathway is indispensable for gaining insight in both proximate and ultimate aspects of its effects. It is also important to note that experimental manipulation may lead to a decrease in maternal fitness, not because the maternal effect is maladaptive, but because the manipulation has forced the system to deviate from the optimal trade-off which may well be different for each individual. Another layer of complexity is added by the possibility that natural selection may have favoured certain responses in the offspring to these maternal effects, to maximize their fitness instead of that of their mother. Despite these difficulties, we will present some examples from avian species that strongly indicate the functional significance of maternal hormone transfer to the offspring, illustrating the concepts discussed above as well as the feasibility of the experimental approach under field conditions.

2. The avian egg as a model

Eggs of all avian species that have been analysed contain in their yolk substantial concentrations of androgens, both testosterone, 5-alpha-dihydrotestosterone and androstenedione, which are much higher than those detectable in the plasma of an embryo or adult bird. Lower concentrations of estradiol and corticosterone are also present (for references see below). In Fig. 2, we report the mean yolk hormone concentrations in freshly laid eggs of one of the most studied species by animal ecologists, the great tit (Parus major). Since these hormones are detected before the stage when the embryo starts its endogenous secretion, as well as in unfertilised eggs, there is no doubt that they are of maternal origin. The presence of maternal androgens in the eggs makes birds an excellent model to study hormone-mediated prenatal maternal effects. The advantage over mammalian species is that the embryo develops outside the mother’s body in a sealed environment, namely, the egg. After egg laying, the mother cannot have further direct influence on offspring prenatal development, with the exception of her incubation behaviour. Thus, egg hormonal provisioning,
being a maternal effect, is likely to be extremely important in these birds.

By far the largest part of this provisioning occurs during a relatively small time window of less than a week, making possible the mapping of the environmental situation to maternal condition and hormone deposition. Furthermore, the fact that the embryo develops outside the mother’s body enables adequate measurement of levels of maternal hormones, as well as the experimental manipulation of these, without requiring interference with the mother. These advantages hold true for oviparous species in general. Indeed, eggs of many vertebrate oviparous species such as fish, [[211]] turtles [[22]] and reptiles [[23]] contain androgens. However, birds produce relatively large eggs, making it possible to study maternal effects for individual embryos. In addition, bird ecology is often well-known and can be studied in the field, facilitating studies of the adaptive value of maternal effects.

By far most of the work on avian egg hormones concerns androgens. Therefore, we will focus on these hormones, although we realize that the biological relevance of other steroids might be equally relevant and may have synergistic effects. We will first review the effects of maternal androgen exposure in avian embryos on behaviour, morphology and immune function, both in the short- and in the long-term. Next, we will discuss the potential adaptive value of maternal androgen deposition, both at the level of within-clutch and between-clutch variation and we will discuss the potential role of androgens in sex allocation. Finally, we will briefly outline potential mechanisms of maternal hormone transfer in birds.

### 3. Effects of prenatal androgen exposure in avian species

Since androgens are lipophilic, and are probably deposited in the egg yolk during the phase of rapid yolkling in the ovary by steroidogenic cells in the follicle wall (see Section 5), it is assumed that the majority of egg androgens are transferred in the yolk of the egg, although this has still to be verified (see below). Therefore, the effects of prenatal exposure to these androgens (from now onwards called prenat(T)) have been tested by injecting androgens, dissolved in vehicle, into the yolk of the egg, and subsequently monitoring chick development and comparing the results with those obtained with eggs injected with vehicle only. This technique is now applied increasingly frequently. When used, it is extremely important that the dose of these injections is 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. This means that the dose employed, together with the endogenous level of hormones in the egg, would result in a concentration that is within the upper limit of naturally occurring levels of that hormone in that species. Such a dose is very much smaller than that used in studies on sexual differentiation, in which injections are administered typically when embryonic development already has taken place for several days: these studies are, therefore, not considered here further. Despite the subtlety of the low dose treatment, clear effects seem to emerge (Table 1), which are discussed below.

#### 3.1. Early competitiveness

In especially altricial or semi-precocial bird species, the chicks are completely dependent on the parents for food, for up to several weeks after fledging. Siblings compete for this food and success in this competition depends on two important factors: first, the time, relative to the siblings, of hatching, since earlier hatching will result in earlier food provisioning and earlier mass gain, providing the chick with

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<tr>
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* Supraphysiological dose.
a size advantage in the subsequent sibling competition. Second, the performance of conspicuous food-soliciting displays, called begging displays. We will summarize the effects of prenatT for these two parameters separately.

In two independent studies with the black-headed gull, a semi-precocial species, prenatT-chicks hatched about half a day earlier than controls [24,25]. This is consistent with the finding that in two other altricial bird species egg testosterone levels are positively correlated with the mass of the musculus complexus,[26,27] which is involved in hatching and begging behaviour, and that experimental elevation of these hormones resulted in an increase in mass of this muscle [26]. PrenatT did not affect hatching time in starlings [28] but these results should be interpreted with some caution because, in this study, the eggs were injected with a dose of testosterone that is 10 times higher than the naturally occurring level in eggs of this species. Although only beneficial effects and no detrimental effects of the treatment on the embryos and chicks were found (see below), and the eggs contain high levels of the pro-androgen androstenedione, the high dose used may well have masked more beneficial effects of naturally occurring levels of yolk androgens.

Surprisingly, prenatT strongly increased time to hatching in a study with American kestrels [29]. Our recent experiment with zebra finches yielded a similar result, [30] although much less so than in the case of the kestrel, where the delay reached several days. We injected whole clutches of zebra finches at the day of clutch completion with either testosterone (500 pg in 5 µl oil, corresponding to the mean difference in androgen levels between clutches laid by females paired with preferred and non-preferred males, see Section 4.2) or 5 µl oil only. Nests were checked daily for egg laying and twice daily for hatching and eggs and chicks were individually marked. Time to hatching in prenatT embryos was 14.0±0.9 days (mean and standard deviation, n=28 eggs from 11 clutches), while it was 13.4±0.6 (n=39 eggs from 13 clutches) for embryos from control eggs (Hierarchical model to account for the nested design of eggs belonging to the same clutch: p = 0.001).

We do not have a methodological explanation for these different effects in different species. In both the zebra finch and the gull study, quite low levels of androgens were injected, while in both the kestrel and in the gull study a combination of both androstenedione and testosterone was injected and incubation took place in the field. The consistency of the results in the gull studies suggests that these different effects on hatching time are not merely a result of random variation, but may reflect species-specific differences in the reaction to early androgen exposure. We speculate that this may reflect species-specific differences in the importance of the speed of development in vulnerable ground-breeders compared to the less vulnerable tree and cliff breeders. Such an explanation could and should be tested by an extensive comparative approach (see Section 6).

PrenatT enhanced the frequency of begging display, which was observed both in standard tests in the laboratory with canary chicks, [31] and during parent–young interactions of gull chicks in the field [25]. In our recent experiment with zebra finches, [30] begging frequency in standard tests was significantly higher in prenatT female chicks compared to control female chicks. This was not the case for the male chicks, perhaps because control males begged at higher frequencies than control female chicks, inducing a ceiling effect for the androgen injection in the males. Such stimulating effects of prenatT on begging are not only restricted to this form of sibling competition for food. In the precocial male domestic chicken, prenatT stimulated the frequency with which chicks chased peers that were given a mealworm as well as the frequency of aggressive pecks (T.G.G. Groothuis, B. Riedstra, and C.M. Eising, unpublished observations).

In the recent experiment with starlings, no consistent effect of prenatT on early begging was found: [28] PrenatT slightly suppressed begging at hatching in acoustic stimulation tests, but not in another test, and no consistent difference was found at Day 5 after hatching. However, the chicks were either tested in isolation, an unnatural situation, or in a group that was first taken from the field to the laboratory, and this may have influenced the results. More importantly, the study used supraphysiological levels of testosterone that may have masked a clear enhancing effect (see above).

The stimulating effects on early chick behaviour may be due to higher circulating levels of androgens in the first few days after hatching when chicks still consume yolk reserves. It may also be due to a priming effect of yolk androgens on androgen production or androgen sensitivity after hatching. Both possibilities need critical testing, since post-natal androgen treatment suppressed begging in the black-headed gull [32]. In kitiwakes, begging is under the control of corticosterone, [33] suggesting that prenatT affects the hypothalamus-pituitary-adrenal axis. However, in the quail prenatT chicks show lower levels of fecal corticosterone metabolites than controls, [34] although this result is difficult to interpret since control chicks hatched from uninjected eggs instead of eggs injected with vehicle only. PrenatT may also stimulate the development of the motor system for begging ([35], see also below) or induce a higher level of hunger motivation. The latter is consistent with the finding that the same treatment promotes alertness and activity in situations in which the chicks are waiting for their parents to return with food [25]. The possibility that this higher activity itself is, due to higher energy expenditure, the cause of an increase in hunger level is unlikely since prenatT did not increase daily energy expenditure [36].

Finally, in the Japanese quail prenatT induced shorter latencies to approach a novel object and to stand up after a tonic immobility test, as well as less vocalizations in an open field test [34]. Similar results have been found in the domestic chicken (T.G.G. Groothuis, B. Riedstra, and C.M.
Eising, unpublished observations). This indicates that prenatT may have a general effect on the regulation of early post-natal behaviour that is not limited to social competition.

### 3.2. Early growth

The increase in food-soliciting behaviour seems effective since in the studied species the group that begged more grew relatively fast in terms of body mass and tarsus length,[24, 30,31] and the timing of opening the eyes, [31] but with one exception [25]. This exception may be due to the fact that in this latter study brood size was reduced because of the experimental design, creating a situation where parents may have had an easy task to feed both experimental and control chicks at a high level. Nevertheless, in that study too prenatT chicks received a greater share of food than their controls in those instances where the parents delivered food items that could be monopolized. This suggests that the effect of prenatT depends on the food situation. The possibility that the effect of yolk androgens on growth is at least partly mediated by its effect on sibling competition is indicated by the finding that chicks from oil injected eggs showed a lower growth rate when reared in nests with chicks from androgen injected eggs than in nests with chicks from oil injected eggs [24].

In another study with bluebirds, [37] prenatT stimulated chick growth too. Two different doses of testosterone were used, of which the lower one induced a slightly higher level of total yolk testosterone than the highest level found in unmanipulated eggs. The low dose enhanced early skeletal growth, and the high dose resulted in higher body mass and skeletal growth around the time of fledging. Finally, prenatT, although in a supraphysiological dose (see Section 3.1) enhanced growth in starling chicks, in the first few days after hatching [28]. Since in this study, prenatT did not enhance early begging behaviour, the results suggest that prenatT can affect growth independently of begging, providing begging was accurately measured (but see Section 3.1). The authors suggest that the higher growth rate in prenatT birds may be causally linked to their somewhat lower begging activity, saving energy [28].

PrenatT may also induce more efficient utilization of nutrients, as has been found for fish larvae, [21] or a different allocation of energy (see Sections 3.3 and 4.1).

In contrast, in a study carried out with the altricial American kestrel prenatT impaired growth [29]. Recalculation of the data in relation to hatching position indicated that this effect may be attributed to the delaying effects of prenatT on hatching time, inducing a severe disadvantage in sibling competition.

### 3.3. Early immune function

In behavioural ecology, the possible immune suppressing effect of androgens is receiving a lot of attention in the framework of sexual selection theory. Such a potential cost of exposure to androgens has been reported for several bird species [38–40]. Furthermore, there is some evidence that the maintenance of the immune system with its large turnover of cells, as well as mounting an immune response, is energetically costly,[41,42] especially for young birds the tissues of whose immune system are still growing [43,44]. Therefore, faster growth induced by prenatT might be at the expense of immune function in circumstances when resources are limited. This effect has been claimed to occur in lizards [45] and quail [46]. However, neither study is definitive. In the first, females were implanted during pregnancy with tubes filled with dihydrotestosterone, while the control females did not receive an appropriate ‘sham’ or control treatment. In addition, the dose was not scaled to approximate naturally occurring maternal levels of the hormone. Although the young of the androgen-treated females showed enhanced growth in parasite free conditions and retardation in growth when challenged by ectoparasites, whereas the young of untreated females did not, overall parasite load or immunological parameters did not differ between the groups. Similarly, in the quail study, the dose of androgens injected in the eggs was not scaled to species-appropriate levels. Again, no overall effect of prenatT on immune function (measured as the T-cell mediated cellular response to a subcutaneous injection with an antigen) was demonstrated. However, prenatT suppressed the immune response in fast, but not in slow, growing birds. Therefore, both studies suggest there may be a relation between immune function and growth mediated by testosterone. A suppressive effect of prenatT on the cellular immune response in the pre-fledging phase was also found in a bluebird study [37]. However, the effect was just not significant for the low dose (and significant for the pharmacological dose), perhaps since sample size of the experimental group was small (n = 5).

An overall suppressive effect of prenatT on immune function has recently been demonstrated by our group in two independent gull studies. The cellular immune response to a standard challenge with a mitogen (PHA) was significantly lower in young chicks hatched from androgen-treated eggs than from oil injected eggs [47,48]. Moreover, the humoural response in 7 days old prenatT chicks to a standard dose of a lipopolysaccharide, containing cell wall fragments of the bacteria *Escherichia coli*, was suppressed too [48]. These results are in line with an experiment with broiler eggs, in which prenatT, although administered relatively late in development (Day 8 of incubation) in a pharmacological dose, suppressed the growth of the bursa of Fabricius in a dose dependent manner [49]. This bursa is the main site for the maturation of lymphocytes.

In both the studies on lizards, [45] quails, [46] blue birds [37] and one study on gulls [47] the data suggest that increased growth, induced by maternal androgens, may be causally related to reduced immune function. However, in the other gull study, [48] immune suppression was induced
without an enhancement of growth, suggesting a direct effect of maternal testosterone on immune function, perhaps via androgen receptors known to be located in the thymus and bursa (for references, see Refs. [37,49,50]).

3.4. Early survival

Survival is a key aspect of fitness. In both the two gull studies mentioned above, survival until fledging was enhanced by prenatal, but this did not reach statistical significance [24,25]. In both studies, prenatal chicks died later after hatching than control chicks, especially so during the first week after hatching, which was statistically significant in the study that used the more sensitive matched pair design [25]. Both studies did not look at possible sex-specific effects on survival. Therefore, we replicated our earlier study following exactly the same design, injection protocol and data collection protocol as in the earlier study, [25] while we additionally determined the sex of the chicks by molecular methods, [48] which enabled us also to match the experimental nests for sex.

While finding no overall effect, mortality until fledging (at the age of 25 days) was significantly lower for males hatching from androgen-treated eggs compared to their controls, which hatched from oil injected eggs (N=16, 6.2% mortality in prenatal males vs. 50.0% mortality in control-males; Wilcoxon Gehan statistic 6.59, p = 0.01). The treatment did not affect female mortality (N=18, 44.4% mortality in prenatal females vs. 33.3% mortality in control-females; Wilcoxon Gehan statistic 0.31, p = 0.58).

So, prenatal had a clear beneficial effect, but only for the male sex. The lack of effect in the females may account for the relatively small effects we reported in earlier studies in which we did not control for sex.

This sex-specific effect is not unexpected. Males in this gull species are 15% larger and likely to be more vulnerable to food shortage [51]. Therefore, the beneficial effects of prenatal on food acquisition via nest soliciting might be more important for male than female chicks in a situation of sufficient food for the smaller sex. The latter was probably the case in our experiment since the brood size was reduced from three to two eggs. Therefore, under more competitive situations females may have profited from prenatal too.

In our recent study on zebra finches ([30], see Section 3.1), post-hatching survival until fledging was clearly higher for prenatal chicks than for controls, irrespective of sex (Fig. 3, data based on daily nest checks). This difference in the pattern of mortality between testosterone (n=28) and oil injected eggs (n=39) was significant (Wilcoxon Gehan statistic: 6.4, p < 0.05). Chicks from oil-injected eggs had a higher mortality in the first three days after hatching. When we took the nested structure of eggs within clutches into account this early effect of treatment was still significant (Wald Chi² = 4.5, p < 0.05). Hierarchical linear model, using the logit link function in Mlwin for binary data, extra-binomial error distribution and parameter estimation by second order penalized quasi-likelihood. Prenatal also enhanced early chick survival under poor food conditions in the starving study [28]. Although intriguing, these results should be treated with caution since the number of chicks that actually died was small. As might be expected based on the effects on hatching time and growth, prenatal induced higher mortality in the American kestrel, [29] which may be completely attributed to its delaying effect on hatching time. This delay probably induced a severe handicap for the later-hatched chick in competition with its older (and now larger) siblings for food delivered by the parents. However, hatching time is not always found to be related to mortality. In the gull study, [25] prenatal chicks and control chicks were deliberately matched for hatching time, while the treatment still enhanced survival. In the starving, prenatal enhanced survival without affecting hatching time. In the zebra finch study, [30] survival was enhanced despite a delay in hatching time. However, in this study, a between-nest design was used, in which all eggs of the same clutch received the same treatment. Consequently, all birds of a prenatal clutch would delay their hatching time, which would not affect sibling competition. This is in contrast to the within-clutch design of the kestrel study. In any case, both the gull and zebra finch study show that the beneficial effects of prenatal are not solely mediated by its effect on hatching time. Its beneficial effect is also not necessarily linked to growth, since in both the gull and zebra finch studies there was no overall effect of treatment on growth (see above).

Why prenatal induced a detrimental effect only in the American kestrel is as yet unclear. The finding is especially intriguing since in this kestrel species, all eggs of the clutch, except the first laid egg, contain high levels of androgens [29]. Therefore, a general detrimental effect of yolk androgens in this species is very unlikely. Perhaps in this species the first egg is not ‘designed’ to contain elevated levels of these hormones. The effect of treatment may
depend on the quality of the egg, such as nutritional factors and immune promoting yolk components, known to vary with laying order [52]. However, the gull studies with opposite results also used androgen injections in first laid eggs that contain relatively low levels of androgens. Another possibility is that the effect of prenatT is sex specific, and that the sex ratio in the first eggs in the kestrels was strongly skewed to the more vulnerable sex. Finally, one aspect of the kestrel study, which was different from the other studies, is that the design (injection of the first laid egg) resulted in an unnatural pattern of yolk androgen concentrations over the laying sequence (after treatment, all eggs had high levels, so disturbing the normal within-clutch pattern). Since the other studies all reported effects that seem to be beneficial for the chick, the kestrel study urgently needs replication.

One other study claimed to have found a possible detrimental effect of yolk androgens. In the house sparrow, the number of hatchlings was negatively related to the level of androgens in the first egg of that clutch [53]. However, this is only a correlation and not experimental evidence. Perhaps, the clutches with higher levels in their first egg might have performed even worse without these elevated levels, which may have been a maternal strategy to compensate for low egg or maternal quality. Furthermore, the results are based on incomplete information of androgen levels in the whole clutch.

### 3.5. Long-lasting effects

Most of the work on the effects of avian maternal androgens has focused on the pre-fledging stage, and often only on the early chick stage. This might be due to the fact that the predominant functional hypothesis for the presence of maternal androgens in avian eggs interprets these hormones as a maternal tool to manipulate sibling competition within the clutch (see Section 4.1). However, yolk androgen levels vary systematically not only within but also between clutches (see Section 4.2). In addition, it is well known that steroid hormones can have long-term organizing effects on the phenotype (see Section 1). Consistent with this effect is the finding that social rank order during feeding in juvenile canaries correlated positively with androgen levels in the yolk biopsies of the eggs from which they had hatched [54]. It has also been suggested that within-clutch variation in yolk levels of testosterone may be responsible for within-clutch variation in song quality in adult male zebra finches, [55] but so far evidence is lacking.

This correlational evidence is now supported by experimental evidence. Our study on the effect of prenatT on begging in the gulls suggests such long-term effects, since the stimulating effect of prenatT on begging behaviour [25] lasted for more than 3 weeks after hatching (Fig. 4). This is far beyond the period of the first few days after hatching in which chicks may still consume yolk from the yolk sac containing elevated levels of androgens.

Effects of prenatT on both social behaviour and plumage have been reported to occur more than several months after hatching. Five-month-old male house sparrows showed a larger secondary sexual character (the black patch on the throat) than controls [56] and in dyadic tests, with weight- and sex-matched controls, both the male and female experimental birds showed a shorter latency to approach a food container on which only one bird could perch. There was, however, no difference in aggressive behaviour or winning interactions [56].

We have found consistent long-lasting effects of prenatT in the black-headed gull. The treatment increased the frequency of threat and courtship display, aggression and also the number of aggressive interactions won, as long as 10 months after hatching [57]. The treatment also induced a further development of the nuptial plumage (the black mask), in early spring [57]. In addition, prenatT lowered juvenile and adult survival [58].

These data indicate that analysis of long-term effects of prenatT is essential for obtaining a comprehensive picture of the functional consequences of avian maternal androgen deposition in the egg. To what extent these long-lasting effects are induced by changing the hypothalamus-pituitary-gonadal axis, the sensitivity to androgens, and/or neural structures remains yet to be understood. The first two possibilities are suggested by the fact that in juvenile and adult gulls threat and courtship display, as well as the nuptial plumage, are under the control of androgens, as is the size of the secondary sexual character in the house sparrow. Recent data indicate that the behavioural effect in the gulls is independent of the effect on the black mask and
due to an increase in sensitivity to testosterone (T. Groothuis and W. Müller, unpublished results). However, although prenatal androgens clearly influence begging, this food-soliciting behaviour may not be directly under the control of post-natal androgen production, [32] but of corticosterone, [33] indicating additional mechanisms.

3.6. Conclusion and discussion

The experimental data indicate that maternal androgens affect a wide range of traits, both in the short-term and in the long-term. The early stimulating effect on begging behaviour and growth would contribute to a more advantageous position for sibling rivalry. Whether the effect on growth is mediated by the effect on begging is yet unclear. Androgens can affect skeletal and neural growth directly (for references, see Refs. [37,54]). However, in some cases prenatalT only stimulated growth after hatching (e.g. Ref. [31]), suggesting a role for sibling competition. It would be of great interest to test the effect of prenatalT on chick growth in nests with and without siblings with whom to compete. This is also relevant since increased growth may bear costs (see Section 4), which should be avoided when not necessary (e.g. without sibling competition).

The results summarized so far are clearly not always consistent. This emphasizes the need for replication studies. There are several potential reasons for the inconsistencies. Recent studies indicate important sex-specific effects and these are not always taken into account so that skewed sex ratios, perhaps even induced by prenatalT (see Section 4.3) may affect the results. In addition, environmental factors or parental quality may vary between experiments and may strongly interact with effects of prenatalT (see Section 4.2). Furthermore, other egg compounds that are important for chick development and interact with androgens (such as carotenoids influencing immune function or egg nutrients influencing early growth) may differ between experiments. If these factors vary systematically over the laying sequence, this would also jeopardize the use of first laid eggs with low doses for injection studies to ‘create’ last laid eggs with higher levels. Moreover, endogenous androgen levels may vary within the same laying position too. Dose of androgen injections are scaled to endogenous levels of eggs of the same species, but often of a different set of clutches. The endogenous levels of experimental clutches may be higher than those to which the dose is scaled to and may easily lead to pharmacological doses, since most applied doses are at the top end of the physiological range. Since yolk biopsies for hormone assays can lead to successful hatching, [31] such biopsies should be used together with injection protocols to avoid this problem. Also, the study of dose–response relationships (see Ref. [37]) would be highly useful here but is almost unexplored.

An important but neglected problem of the injection studies is that avian mothers may deposit levels of androgens in their eggs that are optimal for the current situation. This may mean that each injection would modify yolk androgen levels from this optimum and may lead to detrimental effects. Such a systematic detrimental effect may be avoided by a procedure of cross-fostering the eggs to randomly chosen other pairs; although this would induce a lot of noise in the data, it would remove the possibility of systematic effects which might be misleading. We are currently performing experiments to test this hypothesis of individual optimisation by means of cross-foster studies combined with injection protocols.

Finally, the field lacks the tool of lowering yolk androgen levels or blocking their effects. Flutamide, an androgen receptor blocker, has been applied, [26,49] but has the disadvantage that it will block the effects of endogenous androgen production by the embryo itself too so that the results may be difficult to interpret.

4. Adaptive explanations

We will now explore to what extent the effects of prenatalT, discussed above, may be beneficial for maternal and offspring fitness. Apart from the knowledge about the effects of prenatal exposure to physiological levels of androgens, discussed above, it requires insight into the (variation in) natural occurring patterns of hormone deposition in avian eggs. Consistent patterns in androgen deposition have been found at several different levels: over different eggs of the same clutch (within-clutch variation) and among different clutches of the same species or even female (between-clutch variation). In addition, these patterns, as well as absolute and relative levels of androgens, differ between eggs containing male or female embryos, and differ between species. We will not deal with among species variation in absolute and relative clutch levels of maternal androgens, since this would require a study in its own right. In the following, we will discuss patterns of within and among clutch variation and their possible functional significance, as well as the role of maternal androgens for sex allocation.

4.1. Within-clutch variation

The majority of avian species produces clutches of more than one egg, laid with intervals of one day or more. In many of these species, androgen concentrations in the yolk vary systematically over the laying order (Table 2). This within-clutch variation in androgen deposition has been interpreted in the context of sibling competition, which is strongly related to the phenomenon of hatching asynchrony. Hatching asynchrony occurs when incubation starts before the last egg(s) are laid and as a consequence, last laid eggs hatch later than earlier laid eggs of the clutch. Since parental feeding starts before all eggs have hatched, the later hatched chicks have to compete with older and bigger siblings for food or warmth that is provided by the parents. Thus,
Overview of patterns of androgen deposition over the laying sequence in avian eggs as reported in the literature

<table>
<thead>
<tr>
<th>Within-clutch pattern</th>
<th>Species</th>
<th>References</th>
<th>Variation in pattern related to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase over lay sequence</td>
<td>Domestic canary</td>
<td>[54,68,85,86]</td>
<td>Photo period [68]</td>
</tr>
<tr>
<td></td>
<td>Red-winged blackbird</td>
<td>[26,59]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>American kestrel</td>
<td>[29]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Starling</td>
<td>[27,70]</td>
<td>Maternal age [70]</td>
</tr>
<tr>
<td></td>
<td>Black-headed gull</td>
<td>[24,25,69,73]</td>
<td>Hatching asynchrony [69]</td>
</tr>
<tr>
<td></td>
<td>Black-backed gull</td>
<td>[65,77]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Common tern</td>
<td>[71]</td>
<td>Population</td>
</tr>
<tr>
<td></td>
<td>House sparrow</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cattle egret</td>
<td>[60]</td>
<td></td>
</tr>
<tr>
<td>Decrease over lay sequence</td>
<td>American coot</td>
<td>[61]</td>
<td>Maternal diet [64] sex [101]</td>
</tr>
<tr>
<td></td>
<td>Zebra finch</td>
<td>[62–64,101]</td>
<td></td>
</tr>
<tr>
<td>No overall variation</td>
<td>Tree swallow</td>
<td>[94]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>House wren</td>
<td>[72]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zebra finch</td>
<td>[55]</td>
<td>Unclear</td>
</tr>
<tr>
<td></td>
<td>Great tit</td>
<td>[66,67]</td>
<td>Hormone, [66]</td>
</tr>
<tr>
<td></td>
<td>Japanese quail</td>
<td>[112]</td>
<td>selection line [67]</td>
</tr>
</tbody>
</table>

* The pattern varies according to certain environmental conditions (see last column and text).
* Only last laid egg significantly lower than rest of the clutch.
* Only last laid egg significantly lower than rest of the three-egg clutch.

hatching asynchrony produces a disadvantage for the later-hatched chicks from later-laid eggs. An increase of yolk androgen levels over the laying sequence, present in the majority of species (Table 2), may function as a maternal tool to mitigate the disadvantage of being a later-hatched chick [54,59]. The opposite pattern may function to help the older sibling(s) to out-compete the younger ones in siblicidal species when food is not sufficient to rear the whole brood [60]. Such a form of brood reduction may enhance maternal reproductive success since it would lead to offspring of higher quality, instead of dividing the limited resources over too many young, and to the survival of birds of low quality. We will refer to these explanations as the ‘hatching asynchrony adjustment hypothesis’.

These functional explanations of within-clutch patterns of androgen deposition are consistent with the findings discussed above that prenatT enhances competitiveness, growth and survival in the chick phase in many species. Within that framework, immune suppression may also be seen as an adaptive strategy. As explained above, immune function is energetically costly. For the relatively small, last-hatched chick allocating additional resources to growth, enabling it to compete with the older siblings, may be a prerequisite to survive. Therefore, re-allocating energy from immune function to growth may be an adaptive strategy of making the best of a bad job.

However, the hatching asynchrony adjustment hypothesis might be challenged both on the data presented in Table 2, and theoretical considerations, which we will discuss in this order.

4.1.1. Data from descriptive and experimental studies

A finding that is potentially inconsistent with the hypothesis is that also in two non-siblicidal species in which hatching asynchrony occurs, the precocial American coot [61] and the altricial zebra finch,[62–64] yolk androgen levels decrease over the laying sequence. However, one could argue that brood reduction may be adaptive in non-siblicidal species too when food availability is not sufficient for rearing the whole brood. If so, this would also apply to many other bird species. Moreover, for an optimal adjustment of hatching asynchrony to the need of brood reduction, one would expect that many species have considerable flexibility in how the mothers deposit androgens over the laying sequence. This would mean that one study alone is not sufficient to characterize the species-specific pattern of androgen deposition over the laying sequence. Furthermore, the interpretation of androgen deposition patterns over the laying sequence would only become meaningful when measured together with factors influencing the need for brood reduction, such as food availability, clutch size and maternal quality. This has yet hardly been undertaken (but see Ref. [65]).

Two additional layers of complexity are added by the findings that (1) different androgens [66] and (2) different ‘personalities’ within the same species [67] may show different patterns over the laying sequence. This indicates that our knowledge of understanding within-clutch patterns of androgen deposition is far from complete.

There is some evidence for at least some flexibility in androgen deposition within species (Table 2, last column). The increase of androgen levels over the laying sequence was less pronounced in the domestic canary in clutches produced later in the season [68]. An even more extreme case is that of two selection lines of great tits, one breeding early and one breeding late in the season. The former shows an increase, but the latter a decrease in androgen concentration over the laying sequence [67]. In addition, in the black-headed gull the increase in androgen concentrations over the lay sequence was less pronounced when the estimated hatching asynchrony (indicated by differences in embryo size), was less large [69]. The increase was also less marked in clutches of first year female starlings than in those of older females [70]. Finally, in the common tern, populations differed in the degree of androgen increase over the laying sequence [71]. The first four findings may fit into the hatching asynchrony adjustment hypothesis. Later laid clutches are
often faced with deteriorating food conditions, so that rearing the full brood would be difficult. This would make the compensation of hatching asynchrony by deposition of high levels of androgens to last laid eggs in late broods of canaries and tits less favourable. Similarly, gull clutches with less hatching asynchrony need less compensation while inexperienced starling mothers may benefit from less compensation since they might not be able to rear the whole brood, regardless of season.

Unfortunately, these explanations are all post hoc explanations of descriptive data. Furthermore, in one species in which hatching asynchrony was actually measured, the house wren, the pattern over the laying sequence, as determined in yolk biopsies, was flat, and did not correlate with the degree of hatching asynchrony of that clutch [72]. Therefore, we urgently need data of yolk androgen deposition patterns in relation to experimental manipulation of food availability.

Two such experiments have been carried out. Female black-backed gulls that were given supplementary food in the field during the period of egg production produced clutches that contained lower levels of androgens, while the pattern of androgen levels over the laying sequence was unaffected [65]. Although this fits the suggestion that lower quality eggs are compensated by higher levels of androgens, it is contrary to the hypothesis that avian mothers favour last hatched chicks when food is abundant. In a similar experiment with captive zebra finches, however, mothers on poor quality diet deposited more androgens in their first eggs, supporting the hypothesis that under such conditions mothers should facilitate brood reduction [64]. Clearly, we need more experimental studies in which also other factors are taken into account that influence the position of the last hatched chicks in the sibling rivalry, such as maternal quality, and egg mass and yolk mass that often substantially change over the laying sequence and influence chick survival [74]. Although the majority of studies suggests that avian mothers are not able to adjust within-clutch patterns of androgen deposition to a large degree, switching from an increase to a decrease over the laying sequence is possible, as indicated by the studies on the zebra finch [55,64] and great tit, [67] showing substantial variation within the same species and even within the same female [64].

Finally, the relation between yolk levels of androgens and hatching asynchrony may be confounded by egg quality. As explained above, hatching asynchrony is strongly related to the position of the egg in the laying sequence. Egg quality, measured in terms of egg size, egg mass, levels of carotenoids or antibody concentrations, often decreases in the course of the laying sequence [52,61,75]. This opens the possibility that the increase in yolk levels of androgens in at least some bird species functions as compensation of low egg quality and not of the direct effects of hatching asynchrony.

4.1.2. Theoretical considerations

The hatching asynchrony adjustment hypothesis entails a paradox for species that show an increase in androgen deposition over the laying sequence. Why would mothers produce hatching asynchrony, allowing them to cull the brood size, by eliminating the weakest chick(s), so that brood size becomes appropriate for the available resources, whilst at the same time mitigate the effects of hatching asynchrony by differential androgen allocation? In addition, one could argue that avian mothers can control the degree of hatching synchrony simply by adjusting the incubation pattern alone, without the need for differential androgen deposition. For a solution of this paradox, we have to look at the possible function of hatching asynchrony in birds, for which many hypotheses have been put forward (for a review, see Ref. [76]), and different explanations may be true for different species.

One explanation of this paradox is that hatching asynchrony is an inevitable consequence of the incubation pattern that is adaptive for other reasons. An early onset of incubation during egg laying may protect the egg from predation, or from weather conditions that would decrease egg viability [73]. The compensatory effect of increasing androgen levels in last laid eggs may then be compensation for the adverse effects of early incubation on hatching synchrony. Perhaps birds cannot sit on their eggs for protection without stimulating embryonic development to some degree. This explanation might also explain the relationship between the degree of hatching asynchrony and the increase of androgens over the laying sequence in an open field breeder, such as the gulls, [69] while such a relationship was lacking in house wrens [72]. The latter species breeds in nest holes that provide much more protection to the eggs. Therefore, this species is not forced to start breeding after laying the first egg and it may affect hatching asynchrony by simply varying the incubation patterns, without the need for varying androgen deposition. The open field breeders do not have this freedom and have to sit on their eggs from laying the first egg onwards, and therefore can only minimize hatching asynchrony by increasing androgen deposition in the last laid eggs. A comparative study might be very useful here.

Another possibility for explaining the paradox is that yolk androgens do not really compensate for all the effects of hatching asynchrony. Hatching asynchrony might create a size hierarchy among siblings that might be adaptive for survival of the whole brood by relaxing sibling rivalry. This would allow the parents to selectively feed the most hungry chicks by facilitating nestling identification, or reducing the peak load in food provisioning since the maximal growth rate of the chicks would not occur in synchrony. Increasing levels of androgens in later hatched eggs may then be needed to give these chicks a better chance in the competition with older siblings, without affecting hatching asynchrony and the size hierarchy. This seems indeed to be the case, since the evidence that prenatT influences begging
and sibling competition is much stronger than the evidence that prenatT influences hatching time (Table 1). Furthermore, although prenatT enhances growth, it does not fully compensate for the difference in growth between first and last hatched chicks [24,31].

Finally, it has been argued that prenatT is an additional maternal tool to adjust brood reduction since prenatT would be beneficial only under good food conditions, but detrimental under poor food conditions, because of its possible costs on energy metabolism and immune function [77]. If so, maternal androgen deposition provides the mother with a tool that adjusts brood size to food conditions at the actual time that the food situation for the chicks is relevant, after hatching. However, the available data suggest that prenatT is beneficial even under poor food conditions [28].

4.1.3. Conclusion

Within-clutch patterns of androgen deposition may be adaptive for maternal fitness, but the explanation is more complex than previously assumed and may be different for different species. We clearly need studies that combine the analysis of yolk androgen levels, maternal condition, egg condition, food condition, clutch and brood size and the degree of hatching asynchrony, while taking into account differences between individual mothers and between different androgens. Moreover, more experimental studies, both in relation to egg viability, the need for brood reduction and the advantage of hatching asynchrony, are indispensable to test the possible adaptive explanations. Finally, the long-term effects of prenatT open the possibility that within-clutch variation of androgen deposition provides the mothers a tool to maximize reproductive success by producing different phenotypes, each adapted to different environmental situations (see Ref. [78] for a similar suggestion in relation to within-clutch variation of other factors than yolk androgens).

4.2. Between-clutch variation

Although within-clutch variation of yolk androgen levels has received most of the attention, there are several reasons to believe that between-clutch variation is equally important. First, all eggs contain substantial levels of maternal androgens, not just the last or first egg within a clutch, although this should be sufficient for the hatching asynchrony adjustment hypothesis. Second, some species do not show differential allocation of androgens over the laying sequence. Third, in many studies between-clutch variation in total levels of testosterone, dihydrotestosterone or androstenedione is at least as large or even larger than within-clutch variation in these hormones [53,59,61,68–70, 73]. An overview of studies reporting an influence of environmental factors on overall clutch levels of androgens is presented in Table 3 and this will be discussed below.

4.2.1. Social density

The upper part of Table 3 shows that in the majority of cases an increase in social density (or a decrease in nest box distance) correlates positively with yolk androgen levels. This suggests that deposition of androgens is causally related to the social density a female experiences. However, most of these studies are correlational, opening the alternative possibility that breeding in high density and Table 3

Overview of the effect of environmental or maternal factors on total clutch levels of androgens in various bird species

<table>
<thead>
<tr>
<th>Environmental factor</th>
<th>Effect</th>
<th>Proof</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Social density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nest box distance</td>
<td>Negative</td>
<td>Cor</td>
<td>House sparrow</td>
<td>[80,81]</td>
</tr>
<tr>
<td>Nest box occupation</td>
<td>Positive</td>
<td>Cor</td>
<td>House sparrow</td>
<td>[81]</td>
</tr>
<tr>
<td>Nest intrusions</td>
<td>Positive</td>
<td>Cor</td>
<td>Tree swallow</td>
<td>[94]</td>
</tr>
<tr>
<td>Nest intrusions</td>
<td>Positive</td>
<td>Exp</td>
<td>House sparrow</td>
<td>[80]</td>
</tr>
<tr>
<td>Nest density</td>
<td>Positive</td>
<td>Cor</td>
<td>American coot</td>
<td>[61]</td>
</tr>
<tr>
<td>Nest density</td>
<td>Negative</td>
<td>Cor</td>
<td>Black-headed gull</td>
<td>[73]</td>
</tr>
<tr>
<td>Nest density</td>
<td>Positive</td>
<td>Exp</td>
<td>Black-headed gull</td>
<td>[79]</td>
</tr>
<tr>
<td>Vegetation height</td>
<td>Positive</td>
<td>Cor</td>
<td>Black-headed gull</td>
<td>[73], this paper</td>
</tr>
<tr>
<td><strong>Mate attractivity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ring colour</td>
<td>Positive</td>
<td>Exp</td>
<td>Zebra finch</td>
<td>[62]</td>
</tr>
<tr>
<td>Song quality</td>
<td>Positive</td>
<td>Exp</td>
<td>Domestic canary</td>
<td>[85,86]</td>
</tr>
<tr>
<td>Male testosterone level</td>
<td>Zero</td>
<td>Exp</td>
<td>House sparrow</td>
<td>[53]</td>
</tr>
<tr>
<td>Mate preference</td>
<td>Positive</td>
<td>Exp</td>
<td>Zebra finch</td>
<td>[63]</td>
</tr>
<tr>
<td><strong>Other factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food availability</td>
<td>Negative</td>
<td>Exp</td>
<td>Black-backed gull</td>
<td>[65]</td>
</tr>
<tr>
<td>Early food stress</td>
<td>Positive</td>
<td>Exp</td>
<td>Zebra finch</td>
<td>[93]</td>
</tr>
<tr>
<td>Maternal age</td>
<td>Positive</td>
<td>Cor</td>
<td>Starling</td>
<td>[70]</td>
</tr>
<tr>
<td>Maternal social rank</td>
<td>Negative</td>
<td>Cor</td>
<td>Domestic chicken</td>
<td>[92]</td>
</tr>
<tr>
<td>Laying date</td>
<td>Negative</td>
<td>Cor</td>
<td>Starling</td>
<td>[68,70,81]</td>
</tr>
<tr>
<td>Parasite load</td>
<td>Negative</td>
<td>Exp</td>
<td>Great tit</td>
<td>[66]</td>
</tr>
</tbody>
</table>

Cor, correlational evidence; Exp, experimental evidence.
depositing high levels of androgens are individual characteristics of the female. Furthermore, one study reported a negative relationship between nest density and yolk androgen levels [73]. The authors suggested that in this colonial species aggressive birds, having high levels of androgens and therefore depositing high amounts of these hormones in their eggs, create the low density by themselves by defending larger territories [73]. In the same study, a positive effect of vegetation height on yolk androgen levels was found when corrected for density. Since areas of high vegetation provide cover against predation for the chicks, competition over such areas might be high, which would be consistent with the relation between social density and yolk hormone levels in the other studies. These interpretations are, however, complicated by the following: vegetation height and nest density are strongly positively correlated and this, together with the relatively small data set, makes the results of the multiple regression analysis somewhat unreliable. Since vegetation height by itself is strongly negatively correlated with yolk androgen levels and high vegetation might hamper social interactions, the alternative explanation might be that in this species social stimulation induces higher levels of yolk androgens.

Our recent, unpublished, data support this last possibility. First, the number of vocal and postural displays towards conspecifics other than the partner during 90 min within 24 h after laying of the first egg was more than twice as high in low vegetation (<15 cm height, mean (s.e.m.): 138.1 (20.3)) than in higher vegetation (>15 cm height, 57.7 (20.7)); independent T-test: t = 2.61, n = 16, p = 0.021). Second, average clutch levels of testosterone were also higher in low vegetation (20.01 (1.38) pg/ml) than in high vegetation (14.84 (1.93) pg/ml); Mann–Whitney U-test: U = 236, n = 62, p = 0.008).

Two experimental studies support the interpretation that the number of social interactions is causally involved in androgen deposition. In the black-headed gull, we recently experimentally manipulated social density in captive breeding colonies and found a positive correlation between social density and yolk androgen levels [79]. In another study, pairs of house sparrows were confronted during egg laying with a caged intruder near the nest (or an empty cage) [80]. This type of social challenge is well known to induce a quick surge of testosterone in male songbirds. Treatment affected the time the female spent on the cage, and a positive correlation was found between yolk androgen levels of those eggs that were in the rapid yolking phase during the challenge, and the time the female spent on the cage. This was irrespective of whether the cage contained a conspecific. Perhaps an empty cage in the territory functions already as a challenge that stimulates female testosterone production. Treatment itself only had an effect in a complex interaction with the number of occupied nest boxes around the nest. The exact effects are difficult to assess from the paper, [80] since it presents the data corrected for several other predictors (female levels of testosterone that were not affected by the challenge and nest density that affected female plasma levels of testosterone).

Finally, the relatively low androgen levels in later clutches [53,68,70,81] has been interpreted as an effect of low levels of social interactions too [81]. Social competition is often higher around the production of the first clutch than around the second clutch, since in the first condition new nest sites have to be established while in the second condition eggs are produced in already occupied sites. The social situation is therefore more stable, leading to less stimulation of maternal androgen production.

The evidence presented in the upper part of Table 3 is depicted in a hypothetical causal scheme in Fig. 5, together with two additional findings. First, in house sparrows fecal levels of testosterone from the female during egg laying correlate with testosterone levels of her clutches [68] (for a more extensive discussion of this relationship, see Section 5). Second, there is a positive relationship between social stimulation and androgen levels in birds, including two species listed in this table, the house sparrow [80,82] and the black-headed gull [83] (for a review, see Ref. [84]). The most parsimonious explanation of the observed relationships is depicted with the arrows connecting the different boxes.

The adaptive value of increased androgen deposition in eggs in high density is obvious for (semi-) precocial species such as the coot and gull species. Competition among peers of different broods for food is enhanced in high nest densities. For example, in black-headed gulls, chicks

![Fig. 5. Scheme depicting the relationships between social factors and maternal and yolk testosterone levels in several avian species. Numbers refer to references, arrows between the boxes to the most parsimonious causal relationships.](attachment:image-url)
participate in defending the territory within the colony against intruders that may try to steal food, both during and after feeding bouts, in which food is regurgitated by the parents on the ground. Elevated levels of yolk androgens, stimulating begging, activity, alertness and food competition (Table 1), may positively affect the position of the chick in competition among broods.

However, the function of increased androgen deposition in eggs in high density for altricial species is less clear since the young of these species stay in the nest until fledging. This suggests that the long-term effects of yolk androgens, discussed in Section 3.5, may be important in such cases. Exposure to elevated levels of maternal androgens may prepare the offspring for a higher level of competition after fledging in the juvenile stage, or even in adulthood when it will reproduce in the natal colony.

4.2.2. Mate attractiveness and mate quality

There are five published experimental studies on the effect of mate attractiveness on yolk hormone levels (Table 3, middle part). Female zebra finches have a preference for males with red colour bands over those with green colour bands, perhaps because red is the secondary sexual characteristic on the male bill of this species (for references, see Ref. [62]). In a convincing cross-over design, it has been shown that females paired with red-banded males produce clutches with higher levels of androgens than when paired with green-banded males [62]. In a similar design, female domestic canaries produced clutches with more androgens when exposed to an attractive compared to an unattractive song [85,86]. In both studies, however, attractiveness per se was not measured, but rather was inferred from other studies. We therefore first established, in simultaneous mate-choice tests, the preference of zebra finch females for two male con-specifics. Half of the females were subsequently paired with the preferred male, and the other half with the non-preferred male. The former females deposited more androgens in their eggs than the latter, providing direct empirical support for the conclusions reached in the previous three studies [63].

However, in a fourth study on house sparrows, no support for such differential hormone deposition was found [53]. In this field study, male attractiveness before pair-bonding was manipulated by androgen implants, which are known to affect aggressive behaviour, song rate (courtship) and a secondary sexual signal, the black patch on the throat of males that is lacking in females. Clutches from females paired to androgen-treated males or from females paired to control males did not differ in androgen levels [53]. Unfortunately, the effect of the implants on male testosterone levels during pair formation, on their behaviour, and especially on their attractiveness, was not measured. Furthermore, it is known that testosterone treatment in the period in which it was applied does not affect the black patch of feathers [87]. Therefore, these results are not conclusive while the other studies under controlled condition are all in line with each other.

In all these studies, a positive effect of mate attractiveness on yolk androgen levels was expected for evolutionary reasons. Theory predicts that a female’s investment in a certain reproductive attempt should depend on the fitness return of that attempt [12] and that attractive males will either sire offspring of better genetic quality, or provide benefits such as high quality territories or parental care [88]. Therefore, females are expected to invest more in offspring from attractive mates [88]. The results discussed above seem to support this. However, this explanation assumes that deposition of androgens in the eggs reflects a costly investment for the female. This is yet unclear (see Section 4.4).

We would like to suggest an alternative explanation. This is based on evidence that attractive males may spend more time on mate attraction, obtaining additional mates or extra pair copulations, at the cost of providing parental care. Indeed, males treated with testosterone are doing just that [82,89] and in the experiment with colour banded zebra finches attractive males also provided less parental care [90]. Females may counteract this male strategy by depositing more androgens in their eggs, producing offspring that beg more in order to stimulate paternal feeding.

A potential confound in the above experiments is the finding that females paired with red-banded zebra finches produced a relatively high proportion of sons in their offspring [91]. If females paired to attractive males produce relatively more sons, and if eggs containing male embryos contain higher levels of androgens, than this may explain the relationship between mate attractiveness and yolk androgens. However, at present evidence indicates that eggs with male embryos do not consistently contain higher androgen levels than those containing female embryos, which will be discussed in Section 4.2.3.

4.2.3. Other factors

The effect of food availability on yolk androgen levels (Table 3, last section) might be explained similarly as in the case of mate attractiveness. Under poor food conditions, mothers may benefit from producing offspring that stimulate paternal feeding by enhanced begging. This might be in line with the finding that hens of low social rank and relatively low body weight produce eggs of relatively high levels of androgens [92]. Since maternal body mass is often related to egg mass, it would fit the finding that light clutches contain higher levels of androgens [73].

However, this contrasts the recent finding that mothers reared in enlarged clutches and having lower body mass produced clutches with lower levels of androgens [93]. Perhaps early nutritional stress affects later androgen deposition in a different way than poor food conditions in adulthood that lead to a temporary decrease in body condition. The results are intriguing, indicating how early non-hormone mediated maternal effects may affect the next generation via hormone mediated effects. Also, correlational evidence suggests that older and perhaps more
with age has been found in another study [94]. So, although androgen deposition is costly is not conclusively supported. Furthermore, a suggestion for a weak opposite trend has been found in another study [94]. So, although several studies suggest that when the condition of the mother or food supply is low, higher levels of androgens are found in the eggs, perhaps as some sort of compensation, the evidence is not completely consistent.

Laying date, or photoperiod, seems to affect clutch androgen levels negatively and has been discussed above in the section on social density. Finally, manipulation of ectoparasite load of reproducing great tits affected yolk androgen deposition [66]. As would be expected on the basis of the immunosuppressive effects of prenatT (Section 3.3), mothers in nest boxes infected with parasites produced clutches with (significantly) lower levels of androstenedione and (almost significant lower levels of) testosterone. No effect on dihydrotestosterone was found.

4.2.4. Conclusion

Between-clutch variation in yolk androgens might be at least as important as within-clutch variation. Social density, partner quality, and perhaps food availability that might affect competition among reproducing birds, may influence maternal hormone transfer to the egg. The effect of social density on yolk androgens may prepare the offspring for the level of competition between broods ((semi)-precocial species) and/or for the level of competition in the adult phase when the offspring returns to the natal area (altricial species). Adjusting yolk androgen levels to paternal and food quality may be a maternal tool to adjust the frequency of the offspring’s food soliciting to the level of food provisioning. Experiments testing these functional hypotheses have not yet been conducted.

4.3. Sex allocation

Theory predicts that parents should differentially invest in sons and daughters if the fitness returns from reproducing via sons or daughters differ. These returns can depend on several factors such as the sex ratio in the population, and the costs of producing sons or daughters (especially in sexual size dimorphic species) [95]. Maternal androgens in egg yolk may be related to sex allocation in two ways. First, the same amount of androgens may have qualitatively or quantitatively different effects in both sexes. This might lead to quality differences between male and female offspring. Second, maternal hormone condition, influencing yolk hormone levels, or the latter itself, may determine the sex of the embryo. This is possible for two reasons: first, in birds the female is the heterogametic sex and therefore able to determine the sex of her eggs. Second, meiosis takes place after the rapid yolking phase, just before ovulation, and thus after the deposition of hormones. This makes differential deposition of hormones in eggs according to its future sex unlikely, although both may be correlated to a third factor (for a review on mechanisms of avian sex ratio adjustment, see Ref. [96]).

Some sex-specific results of prenatT (see Table 1) support the first pathway, opening the possibility that maternal androgen deposition does play a role in sex allocation. The second possibility requires differences in hormone concentrations between eggs of the two sexes. The relevant data from the literature are summarized in Table 4, indicating mixed results. A large difference in testosterone and androstenedione concentration (larger in male eggs) and estrogen and dihydrotestosterone concentration (larger in female eggs) was found in a highly sexual dimorphic species, the peafowl [97]. However, the eggs used in this study had been incubated for ten days. At this time endogenous sex-specific production of these steroids by the embryos already has started in related avian species [98, 99] and may have influenced yolk levels of these hormones. Furthermore, in this highly sex dimorphic species sex-specific growth of the embryos may have led to sex-specific rate of yolk consumption and hormone utilization [100]. So these results are inconclusive.

Three other studies show differences in androgen levels between eggs containing male or female embryos in a much earlier stage of development [75,92,101]. In all these studies no overall effect of embryo sex was demonstrated, but only an effect of embryo sex in interaction with other factors. Each study provides its own, post hoc, functional explanation for this sex-specific hormone deposition pattern and

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Table 4
Overview of the results of available studies on differential deposition of maternal androgens in eggs containing male or female embryos

<table>
<thead>
<tr>
<th>Sex difference</th>
<th>Species</th>
<th>Reference</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta T$, A4 $&gt;$ $\delta T$, A4; $\delta$DHT, E $&lt;$ $\delta$DHT, E</td>
<td>Peafowl</td>
<td>[97]</td>
<td>Endogenous production?</td>
</tr>
<tr>
<td>$\delta T$ $&gt;$ $\delta T$</td>
<td>Domestic chicken</td>
<td>[92]</td>
<td>Interaction maternal social rank</td>
</tr>
<tr>
<td>$\delta T$ $&lt;$ $\delta T$</td>
<td>Zebra finch</td>
<td>[101]</td>
<td>Interaction diet and lay sequence</td>
</tr>
<tr>
<td>$\delta T$ $&lt;$ $\delta T$</td>
<td>Black-headed gull</td>
<td>[75]</td>
<td>Interaction lay sequence</td>
</tr>
<tr>
<td>$\delta T$ $=$ $\delta T$</td>
<td>Domestic canary</td>
<td>[54]</td>
<td></td>
</tr>
<tr>
<td>$\delta T$, A4, DHT $=$ $\delta T$, A4, DHT</td>
<td>Black-backed gull</td>
<td>[65]</td>
<td></td>
</tr>
<tr>
<td>$\delta T$, A4 $=$ $\delta T$, A4</td>
<td>Domestic chicken</td>
<td>[100]</td>
<td></td>
</tr>
</tbody>
</table>
needs therefore follow up studies. Nevertheless, it appears that the level of yolk androgens alone does not determine the outcome of meiosis that determines the sex of the future embryo. However, none of the studies really replicated the peafowl study at an earlier stage of embryonic development by measuring several androgens and estrogen. Therefore, it might still be possible that the ratio of the different hormones influences sex determination of the egg.

Three other studies did not find sex-specific androgen levels (Table 4). However, the canary [54] and chicken study [100] only looked at an overall sex effect. The black-backed gull study [65] did not use laying order, sex, and their interaction as predictors simultaneously in the same model, like the other gull study that did find a sex effect [75]. Clearly, more studies are needed here but the available data do suggest a promising avenue for further research. A potential problem in descriptive studies is that incubation of the eggs is required for embryo development sufficient to enable determination of its sex, but yolk levels of androgens have been shown to decrease with incubation time [100, 102]. Such a decrease may be due to androgens entering the albumin and, in a later stage, utilization by the embryo. Analysing the whole egg, and not only its yolk, as early in the incubation process as possible might be a valuable research strategy.

An obvious experimental approach would be to manipulate maternal hormone state during egg production. Recently, it was reported that female spotless starlings, implanted with testosterone, produced a higher proportion of sons in their broods than control females, probably as a consequence of a shift in the primary sex ratio [103]. Clearly, more studies are needed here but the available data do suggest a promising avenue for further research. A potential problem in descriptive studies is that incubation of the eggs is required for embryo development sufficient to enable determination of its sex, but yolk levels of androgens have been shown to decrease with incubation time [100, 102]. Such a decrease may be due to androgens entering the albumin and, in a later stage, utilization by the embryo. Analysing the whole egg, and not only its yolk, as early in the incubation process as possible might be a valuable research strategy.

A final note should be made about the potential influence of maternal androgens on sexual differentiation. This topic requires the study of long-term effects that has, as yet, only been undertaken in a few studies (see Section 3.5) [56–58]. Neither of these found sex-specific effects of prenatT. Effects of yolk androgen on sexual differentiation are not expected to be large. In birds, the male sex is the default sex, and females differentiate because of the action of estrogen [104]. This might be the reason why estrogen levels in the yolk of freshly laid eggs are so low [54,71,73,97]. Indeed, the effect of PrenatT on the sexual character in 5-month-old house sparrows (see Section 3.5) only occurred in the male sex and did not induce such a character in females [56].

4.3.1. Conclusion

Deposition of maternal androgens in avian species might be important for sex allocation. These hormones seem to have sex-specific effects that should more often taken into consideration. Sex ratios can change with environmental circumstances and within the laying sequence. Therefore, variable sex ratios might explain inconsistencies in the effects of maternal androgens on the offspring between different studies. Moreover, androgen levels differ in eggs containing male and female embryos, although in a complex relationship with other factors. Avian maternal hormones might be involved in sex determination, but probably not in sex differentiation.

4.4. Costs and trade-off

So far, we have emphasized the possible benefits of elevated levels of yolk androgens for the mother and chick. However, if high levels of androgens are associated with no costs, then why would not all clutches contain high levels of these hormones? The obvious answer is that there probably are costs, either directly for the chick (with indirect consequences for maternal fitness), or directly for the mother (which may result, indirectly, in costs for the offspring), or both. Because of the sex-specific effects of the yolk androgens, detrimental effects in one sex may constrain hormone deposition in eggs of both sexes, unless avian mothers can differentially allocate these hormones according to the sex of the egg (see above).

High levels of yolk androgens seem to suppress cellular and humoral immune function in the chick (Table 1, see Section 3). Since the chick is vulnerable to infectious diseases, because the humoral part of the immune system develops only gradually over the first weeks of life, [105] this might be a substantial cost for the chick.

One could argue that if this cost is mediated via androgen receptors in the immune system, birds could easily avoid these costs by down regulating these receptor populations. However, the development of the immune system, and mounting an immune response, is probably energetically costly (see Section 3.3), and the bird is then forced to trade-off the costs of immune suppression against allocation of energy to growth. In such a scenario suppression of immune function may then be favoured in fast hatched chicks for which fast growth is crucial, enabling them to survive sibling competition with larger siblings. However, increased growth rate, due to yolk androgens, might be beneficial in the short-term, but have long-term costs [106]. Both long-term suppressive effects on immune function and long-term costs of fast early growth might explain the relatively high mortality in juvenile and adult gulls from androgen injected eggs,[25,58] but this has not been tested as yet.

Yolk androgens may be costly when they would enhance basic metabolic rate, or daily energy expenditure due to higher activity. In one study on black-headed gulls, no evidence for this was found [36]. Elevated levels of yolk androgens may increase, via stimulation of activity and begging, the probability of predation, but no evidence for this has yet been demonstrated. Finally, elevated levels of yolk androgens in the whole clutch may lead to detrimental levels of sibling competition. Only one study compared
broods in which all eggs had been injected with androgens with broods in which all eggs received oil injections [30]. Mean growth rate for both sexes and survival were not lower in the androgen injected broods [30]. In conclusion, yolk androgens do probably impose a cost for the chick in terms of suppressed immune function and long-term survival, while the evidence for other costs could not be demonstrated or have not yet been investigated. These costs may explain why not all clutches contain high levels of androgens. For optimal androgen deposition, avian mothers should trade-off this cost against the benefits discussed earlier. The outcome of this trade-off is likely to depend on the disease environment of the chick, and the need for short- or long-term enhanced competitiveness in the offspring, as well as possible direct costs for the mother. Therefore, the evaluation of fitness consequences of manipulation of yolk androgens should take into account both maternal and environmental conditions, as well as a wide array of traits in the offspring.

The possibility of direct costs for the mother assumes that the deposition of high levels of the hormone in the egg is only possible if the mother too is exposed to high levels of this hormone herself. This is currently the most parsimonious hypothesis, and will be further discussed in Section 5.

Exposure to long-term elevated levels of testosterone can have a wide array of costs in avian males,[13,107] but similar studies on avian females are only just beginning to emerge. The most obvious cost of the hormone in females around egg laying might be the interference with reproduction. Testosterone treatment of females around egg laying can delay clutch initiation,[103,108,109] clutch size, [109] fledging success of the chicks ([103], but see Ref.[108]), but does not seem to affect incubation behaviour [108,109]. However, all these effects have been found with testosterone implants inducing elevated levels of the hormone for at least several weeks. Although in these studies levels were within the physiological range, they might not reflect endogenous levels that are normally needed for elevation of androgen deposition during the stage of egg production. Blood- and fecal-levels of testosterone fluctuate strongly in the course of egg laying,[68,110] and perhaps short-lasting peaks during the most rapid phase of yolk formation may be sufficient for normal androgen deposition in the egg, without inducing the costs as discussed above. It is therefore essential that we learn more about the proximate mechanism of androgen deposition in the egg.

5. Passive transfer or independent pathways?

A core but yet unanswered question is whether or not the avian mother can deposit her androgens in her eggs independently from her own blood levels of these hormones. If not, androgen deposition might be constrained by the regulation and effects of female androgens on the female herself. If these effects involve costs, androgen deposition may be viewed as maternal investment and as an important part of the trade-off between current and future reproductive success. Nevertheless, patterns of hormone deposition are often referred to in the literature as allocation patterns, suggesting some sort of active control of deposition by the female. It is clearly of great importance to integrate the functional-evolutionary approach with knowledge about the mechanism of hormone deposition.

Two studies suggest that yolk levels of hormones reflect maternal blood levels of these hormones, since a positive correlation between egg levels and fecal or blood levels was found [68,79]. In three other studies, a negative correlation was found [65,80,86]. However, in Ref. [65], maternal blood was collected after clutch completion, and the blood levels may not have been an accurate indication of blood levels during egg formation. In Ref. [80], the negative correlation was found in a statistical model that included several other predictors, including breeding density and experimentally induced social stimulation that both affected yolk androgen levels. The result is therefore hard to interpret. In two other studies, females were treated with hormone implants during egg formation, and the yolk levels of the hormone were higher than in those of control females (estrogen, [111] testosterone [108]). However, this evidence only demonstrates that hormones from the maternal circulation may enter the yolk when blood levels are strongly and continuously elevated, but not whether it actually occurs under normal conditions during egg laying. In addition, these results seem to be in contrast to the finding that only about 0.1% of radioactive testosterone, injected intra-muscularly in the female chicken during egg formation, was detected in the yolk after egg laying [112]. Although this result might be due to the fact that only one injection was given, and much of the hormone was rapidly excreted with the feces, other evidence supports the view that circulating steroid hormones do not easily enter the yolk. In avian species, corticosterone is produced by the adrenals and not by the follicles and very likely only enters the yolk from the maternal blood circulation. Basal blood levels of corticosterone are several times higher than that of plasma levels of androgens, but egg yolk contains much lower levels of corticosterone than androgens (e.g. Ref. [17], Fig. 2).

The issue of the correlation of androgen levels between maternal blood and yolk has also some methodological difficulties. In adult birds, plasma levels can substantially fluctuate due to social challenges (see Section 4.2), while yolk levels fluctuate with incubation time [100,102] and layer of the yolk,[102,112,113] which are deposited in concentric circles. It has been argued that the latter would influence the timing of androgen exposure of the developing embryo, but since the layers seem to mix completely after some incubation, this is probably not the case.

Some support for hormone deposition independent of maternal levels comes from the finding mentioned earlier that levels of estrogen are extremely low in the egg yolk,
despite the fact that estrogens are even more lipophilic than androgens and are elevated in maternal blood during egg formation. In a lizard species, the ratio androgen/estrogen in the egg is substantially different from that in the female circulation, [114] but similar data for avian species are currently lacking. These data open the possibility for independent regulation of maternal and yolk hormone levels, unless egg yolk contains enzymes converting the hormones to other metabolites.

Might female birds possess the physiological mechanism for independent regulation of hormone deposition in the egg and of their own plasma levels? One simple mechanism would be that sex steroid hormones in the yolk come from the ovaries, while those in the maternal circulation are mainly provided by the adrenals. Avian adrenals possess at least some capacity to produce androgens, [115] but it is unlikely that this adrenal contribution to plasma levels of androgens is more important than that of the ovaries. But other possible mechanisms for independent regulation are also conceivable. Although limited, the available evidence for bird species suggests that differential steroidogenesis takes place in the follicle wall in three different cell layers [116–118]. In theory the outer cell layer, the theca externa, might produce hormones for the maternal circulation, while the cell layers closest to the follicle, the theca interna and the granulose cells, produce hormones for the yolk. The hormone production of these cell layers might then act independently from each other with their own dynamics of receptor expression for the gonadotrophine hormones. However, available evidence indicates that the granulose cells mainly produce progesterone, the theca interna mainly androgens, and the theca externa estrogens [116–118]. If so, this renders independent regulation unlikely. This might also explain the ratio of steroid hormones in the blood as discussed above. Androgens are produced by the cell layer that is more close to the follicle than the estrogen producing cells. Corticosterone is produced outside the ovaries by the adrenals and can only enter the yolk by diffusion.

This does not mean that hormone levels in maternal plasma and in her yolk should correlate. There are strong indications that in several bird species hormone production shows large and systematical variation in the course of follicle maturation, while this production might also depend on the order in which the follicles develop, e.g. Refs. [113, 117,119]. Since plasma levels will depend on the total hormone production of all follicles, while that of a particular egg on only its own follicle, plasma and yolk levels may show different profiles. By influencing the stage of maturation of her different follicles mothers may have the potential to influence plasma levels independently from yolk levels. In addition, by depositing more yolk into the follicle, more hormone is deposited as well. Although this will not change the hormone concentration per amount of yolk, it may still influence the embryo. Whether hormone concentration, or total amount of deposited hormone, irrespective of yolk mass, is the most biological relevant entity remains to be seen.

So, although it is most parsimonious to assume that the deposition of maternal androgen in the yolk is not regulated independently from blood levels of these hormones in the maternal circulation, a mechanism for independent control is conceivable. It is also to be expected that such a mechanism has evolved in case it would strongly affect maternal fitness. Unfortunately, convincing data both on the mechanism of hormone deposition and the fitness advantage of independent control are lacking at the moment.

6. Concluding remarks

By reviewing the literature of the rapidly evolving field of avian maternal hormones, we have shown that avian mothers deposit substantial amounts of androgens in their eggs, which have clear and often beneficial effects on the offspring. Based on this, we have discussed several plausible hypotheses for the adaptive value of the systematical variation in androgen deposition within and among clutches, and the involvement of maternal androgens in sex allocation. However, the field, being relatively new, suffers from the use of post hoc explanations, inconsistent results, the lack of replication, methodological difficulties, and the cursory knowledge of the relevant physiological mechanisms. Nevertheless, we hope to have demonstrated both the value of an evolutionary and ecological approach, as well as the value of the avian model, for the study of hormonally mediated maternal effects. Correlational evidence indicates that in humans too maternal androgens affect the offspring, [120] and the time is ripe to analyse these effects also from an adaptive perspective.

The surge in interest in avian maternal hormones started only 11 years ago with a paper of Hubert Schwabl on the presence of steroid hormones in two bird species [54]. It is therefore not surprising that many questions are as yet unanswered. At the moment, the within-clutch variation of maternal androgens is often discussed in relation with the phenomenon of sibling competition. But as we have shown, more data are needed in relation to factors that influence actual sibling competition, such as the degree of hatching asynchrony, incubation pattern, food quality and availability, and maternal condition. Furthermore, alternative explanations, perhaps different for different species, are possible. Among clutch variation in yolk androgens appeared to be substantial and experimental testing of its functional significance has not yet been undertaken. In addition, the possible long-term effects of maternal androgens on behaviour and fitness need further study, as they may have important implications for individual differentiation, local adaptation, and sexual selection. They open also the possibility that the effects are trans-generational, but this has not yet been studied. Furthermore, the role of
yolk androgens in sex determination and allocation are promising avenues for further research.

Given the substantial number of different avian species of which we now have data on yolk androgen levels, a comparison of relative and absolute levels of androgens in relation to life history traits at the species level might reveal important new insights into the function and evolution of yolk androgens. Obvious traits to examine are: developmental time (precoical versus altricial species, colonial species with synchronous fledging versus non-colonial species with asynchronous fledging), degree of sibling competition (siblicidal versus non-siblicidal species, species producing clutches of one egg versus clutches with more eggs), degree of sexual dimorphism, incubation pattern and type of nesting site (influencing the need for early incubation to prevent decrease in egg viability, limiting maternal possibility to minimize hatching asynchrony), and the risk of infectious diseases. Different androgens may differ in their relation with such traits, revealing the different functions of these androgens. Some species like precoical birds have relatively high levels of androstenedione (A4) in their eggs, in contrast to many altricial species, of which some have relatively high levels of dihydrotestosterone (DHT). A4 itself is biologically not very active, but may function as a source for other active metabolites in large eggs, since it can be converted to the more active compounds estradiol and testosterone. Testosterone, in turn, can be converted to estradiol and the active androgen DHT. Most studies tend to focus on testosterone, but the various androgens do not always correlate well (e.g. Ref. [73]) and without further knowledge of the function of the different hormones the focus on testosterone is a bit arbitrary. In addition, analysing levels of several androgens without a priori expectation might increase the change on type II errors. Injection experiments with different (combinations of) androgens may be of great help here.

Finally, knowledge of the interplay of maternal androgens with other pathways for maternal effects might strongly improve our understanding of androgen-mediated maternal effects. These other pathways include the transfer of other hormones and immune relevant substances such as antibodies and carotenoids to the egg that may enhance or counteract the immune suppressive effects of androgens.

The field also urgently needs studies on the physiological mechanisms underlying hormone transfer and the effects of maternal androgens on the phenotype, since the field has become dominated by behavioural ecologists, interested in function and evolution of hormonally mediated maternal effects. Evolutionary ecologists are interested in understanding selection pressures that account for maternal effects, often assuming that the details of the physiological mechanism would not affect their interpretation. However, the use of terms as ‘investment’ or ‘allocation’ for female hormone deposition, which is frequent in the literature, is not (yet?) justified, unless we know more about the proximate mechanism of hormone deposition. We also need to know when and which tissues in the embryo are androgen sensitive, and to what extent the embryo is able to convert androstenedione and testosterone to other hormones, and whether the yolk itself may contain the relevant enzymes for this. Hopefully, this review will stimulate neuroendocrinologists to analyse the physiological mechanisms underlying maternal androgen effects in birds, leading to a much more integrative approach in this field. Finally, the technique of determining yolk androgen levels and injection of androgens in the egg is now widely used, but unfortunately sometimes without sufficient endocrinological background, hampering adequate hormone assays, proper scaling of the injected dose, and correct interpretation.

Given the pronounced effects of yolk androgens on chick development, it is indeed almost inevitable to conclude that this hormone deposition is shaped by natural selection and not just an epiphenomenon of female hormone and egg production. The finding that this deposition is different between two selection lines of great tits suggests that this disposition has a genetic basis on which selection can act [67]. However, it is possible that avian androgen deposition is a correlated trait of the female reproductive system, and that selection has acted on the latter and not on the former. In that case, the effects of maternal androgen deposition in the egg, although beneficial, may be not an adaptation but an exaptation (see Ref. [121] for a discussion of this concept in relation to hormones and avian life history traits). Again, physiological studies can help here, indicating whether or not androgen transfer to eggs involves a specially evolved system. But in any case the results to date strongly indicate that avian mothers prepare their offspring via the transfer of maternal androgens to the conditions they will encounter after hatching.

A final point relates to the potential conflict of interest between mothers and their offspring. Adaptive maternal effects promote Darwinian fitness of the mother, not necessarily the offspring, who are not complete genetic replicates of them. Mothers may try to increase their fitness by rearing the full brood, at the cost of maximal fitness perspectives of an individual chick. In times of food shortage, parents may even benefit by culling the number of offspring to the actual food situation, to rear fewer young of better quality, at the cost of surplus young. This gives rise to two questions: does evolutionary pressure on the offspring exist to (1) enhance the sensitivity to maternal androgens in order to enhance its beneficial effects? (2) to produce its own androgens independently of the mother? There is some evidence for the first possibility. Zebra finch chicks show a transient display of androgen receptors in the hindbrain (that is probably involved in begging behaviour) before the time of sexual differentiation. This is interpreted as being the result of co-evolution of the production of these receptors with maternal hormone transfer [35]. The finding that, in several bird species, embryos of both sexes produce substantial levels of androgens [98,99] may be interpreted
as support for the second possibility. Nevertheless, there are two complications that might limit the possibilities of the embryo in this respect. First, effects of androgens are known to depend on different sensitive phases, and maternal androgens, being present very early in development, may well affect the embryo in a different way than the endogenously produced androgens that can only be produced after development of the gonads and or adrenals. If so, the embryo would not be able to compensate low levels of maternal androgen deposition. Second, the optimal androgen exposure may depend on environmental factors such as the level of sibling competition (position in the hatching sequence, food availability, disease environment) of which the embryo has no information yet. This would give the mother a dominant position in the parent-offspring conflict. The analysis of hormone-mediated maternal effects within the framework of parent-offspring conflicts has not yet been undertaken.

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