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An experimental study on the causes of sex-biased mortality in the black-headed gull – the possible role of testosterone

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Summary

1. During early development male offspring in avian species often suffer from enhanced mortality compared to female offspring. This has been attributed to different nutritional requirements, as sex-biased mortality has been reported particularly in sexually size-dimorphic species. However, other traits of the male phenotype, such as the embryonic hormone profile, have been suggested to contribute to this male disadvantage. In particular the negative effects of sex steroids on immune function may be causally involved.
2. We investigated the role of testosterone in the expression of male phenotype disadvantage through an experimental reduction of the availability of testosterone receptors by *in-ovo* injection of an anti-androgen (Flutamide©). Experimental nests contained a male and a female chick hatching from control treated eggs and a male and a female chick hatching from Flutamide treated eggs.
3. Male-biased mortality occurred in control chicks at a stage where the sexes did not yet differ in their growth pattern, suggesting that sex-specific nutritional requirements are not necessary for male-biased mortality to occur. Control males and control females did not differ in their cell-mediated immunity (CMI). This renders it unlikely that the observed skewed mortality was due to sex-specific differences in the CMI.
4. Treatment of the eggs with flutamide antagonistically affected males and females. Flutamide treatment positively affected male development in particular through an enhanced growth rate, indicating that testosterone is involved in the expression of the male phenotype disadvantage. Female chicks hatching from Flutamide treated eggs were disadvantaged in growth and CMI. The possible pathways of this sex-specific effect of Flutamide are discussed with regard to differential consequences of blocking the beneficial effects of maternal androgens and the interference with the process of sexual differentiation in males.

Key-words: anti-androgen, brood size, Flutamide, immunity, PHA.

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Introduction

Male-biased mortality occurs during early postnatal development, especially in vertebrate species where males are the larger sex at maturation (e.g. Clutton-Brock, Albon & Guinness 1985; Røskraft & Slagsvold 1985; Griffiths 1992). It has been suggested that male development requires more resources, enhancing vulnerability to food shortage and causing greater postnatal mortality of the larger sex. This hypothesis is supported by the fact that the extent of male-biased mortality correlates positively with the degree of sexual size dimorphism (Clutton-Brock *et al.* 1985). Furthermore, it increases under adverse environmental conditions where the risk of food shortage is greater (Røskraft & Slagsvold 1985; Nager *et al.* 2000). The role of sexual size dimorphism in sex-biased mortality is emphasized further by the fact that when females are the larger sex,

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mortality is increased specifically in females (Torres & Drummond 1997; Dijkstra, Daan & Pen 1998). However, it is also likely that other characteristics of the male phenotype contribute to the observed mortality pattern, as male-biased mortality has been reported when nutritional circumstances posthatching were optimal (Nager *et al*. 1999).

In birds, the development of phenotypic differences between males and females, the process of sexual differentiation, begins early in embryonic development. Due to the sex-specific production of gonadal hormones the individual's physiology, endocrinology, immunity and (postnatal) behaviour is organized sex-specifically (Balthazart & Adkins-Regan 2003). In several studies it was found that male embryos produced more testosterone than females during the period of sexual differentiation (Woods, Simson & Moore 1975; Tanabe *et al*. 1979; Ottinger, Pitts, & Abelnabi 2001), but other such work failed to find this (Tanabe, Saito & Nakamura 1986; Schumacher, Sulon & Balthazart 1988). Nevertheless, in all species studied so far, females produce more oestrogens than male embryos, causing consistent sex differences in the ratio of plasma levels of testosterone to oestrogens [Woods *et al*. 1975; Tanabe *et al*. 1979; Tanabe *et al*. 1986; Schumacher *et al*. 1988; Ottinger *et al*. 2001; but see also Tanabe, Takashi & Nakamura 1983 (after the period of sexual differentiation)]. These differences in endocrine state during embryogenesis may be causally involved in the expression of lower immune function in males, as observed for vertebrate species (reviewed in Grossman 1985; Olsen & Kovacs 1996; Klein 2000). In birds, sex differences in immune function have been shown to be present as early as during the chick phase (Fargallo *et al*. 2002; Müller, Dijkstra & Groothuis 2003; Tschirren, Fitze & Richner 2003). However, whereas several studies have examined the effects of sex steroids on immunity through hormone manipulation in adulthood, the question of whether prenatal manipulation of hormone exposure affects postnatal immunity and thus mortality has rarely been addressed.

We hypothesize that sex differences in prenatal hormone exposure may organize the immune system in a sex-specific way, potentially causing sex differences in postnatal survival probabilities in our study species, the black-headed gull *Larus ridibundus*. Gulls are sexually dimorphic (adult males about 12–15% larger compared to adult females) and male-biased mortality during the nestling period has repeatedly been reported (e.g. Sayce & Hunt 1987; Griffiths 1992). We therefore investigated whether the ratio of (endogenously produced) testosterone to oestrogen plays a role in the expression of the male phenotype disadvantage. Hence, we manipulated the embryonic testosterone exposure by a reduction in the number of available testosterone receptors, induced by injection of an anti-androgen (Flutamide©). Sex-biased mortality occurs more commonly under harsh conditions (Røskraft & Slagsvold 1985) and when egg quality is reduced (Nager *et al*. 1999). We therefore increased the brood size by one, with each brood being composed of one male and one female chick of each treatment, and selected second- and third-laid eggs that are of lower quality (e.g. Blount *et al*. 2002).

We expected male-biased mortality to occur in the control situation (sham-treated eggs). If differences in food demand are the cause of this skewed mortality pattern, reduced male survival should occur at the time when the sexual size dimorphism develops. Sex differences in the survival probability before that time should be attributed to other aspects of the male phenotype, because there are as yet no differences in metabolic rate (Eising *et al*. 2003). We investigated the cell-mediated immunity (CMI), as differences in survival probability between the sexes may be a consequence of sex differences in immunity.

We expected the male phenotype disadvantage, causing reduced male survival, to be lower in the Flutamide-treated group. The effect of Flutamide for female chicks may be more difficult to predict, as female embryos particularly experience higher levels of oestrogens (Woods *et al*. 1975; Tanabe *et al*. 1979; Tanabe *et al*. 1986; Schumacher *et al*. 1988; Ottinger *et al*. 2001). They might thus be less affected by Flutamide, as the development of the female phenotype is dependent mainly on oestrogens (reviewed by Balthazart & Adkins-Regan 2003).

However, in both sexes Flutamide is likely to block the effects of maternal androgens, which are known to be present in substantial amounts in gull eggs and positively affect chick growth (Schwabl 1996; Eising *et al*. 2001). Thus the net effect of Flutamide might be detrimental in females by reducing the beneficial effects of maternal testosterone on chick development, but positive for males because the negative effect is counterbalanced by the induction of a more female-like phenotype.

Material and methods

STUDY SPECIES AND POPULATION

The black-headed gull is a sexually size-dimorphic species (adult males about 15% heavier and skeletally larger than adult females); their clutch typically contains three eggs (Glutz von Blotzheim & Bauer 1982). Fieldwork in 2002 was conducted in two colonies of about 300–500 breeding pairs situated at the Northeast coast of the Netherlands. During the laying period nests were checked every day for freshly laid eggs and each egg was marked with a non-toxic marker for its position in the laying order (A for first-, B for second- and C for last-laid eggs) and the date of egg laying. On the day of clutch completion either the B or C egg of a clutch was selected for the experiment and taken to the Zoological Laboratory, where egg treatment and incubation took place. All clutches that provided eggs for experimental nests (donor nests) still contained two eggs.

EGG TREATMENT

Experimental eggs were weighed to the nearest 0·1 g and the yolk was either injected with 50 µL of vehicle (sterile cold-pressed sesame oil) or 50 µL of vehicle containing 0·3 mg Flutamide (for a detailed description of the injection procedure see Eising *et al*. 2001). Chicks hatched from Flutamide injected eggs will subsequently be referred to as Flu chicks (Flu males/females), chicks from oil-treated eggs as control chicks (control males/females). Flutamide has an anti-androgenic function by blocking androgen receptor activity. We selected eggs laid later in the laying sequence as these eggs are known to be of lower quality (e.g. Blount *et al*. 2002). Furthermore, these eggs contain high levels of maternal testosterone (on average 13·2/15·1 pg testosterone/mg yolk, B/C eggs, respectively; Eising *et al*. 2001). The average yolk mass is 9·0 g and the dose (0·08 µmol per egg) used in this experiment is similar to those used by Lipar & Ketterson (2000) and Burke (1996). After treatment, eggs were placed in the incubator for the next 20 days.

EXPERIMENTAL SET-UP

About 2 days before the estimated hatching date, eggs were checked for embryonic development. All well-developed eggs were placed into natural nests, allocating three eggs of the same treatment and same laying date in one nest. The natural eggs of these nests were used to refill the donor nests to the modal clutch size of three, matched for laying date. Experimental nests had been surrounded by mini-enclosures 1 day before placing the eggs, which were made of opaque mesh and measured about 1·5 m in diameter and about 40 cm in height to facilitate the adoption procedure. All mini-enclosures were situated within larger enclosures (wire mesh, 40–50 cm high) varying in size from 30 to 100 m^2. Enclosures contained from 10 up to 15 nests and enabled us to follow chick development when the mini-enclosures were removed after the parent chick bond was established (5–7 days after cross-fostering).

Whenever a chick started to hatch, we marked the bill with a non-toxic pen in order to record which chick came from which particular egg, in case more than one chick of the same clutch hatched on the same day. On the day of hatching (day 0), all chicks received a numbered colour band for individual identification. A small blood sample (20 µL) was taken from the ulnar vein and stored in 100% ethanol. Within 24 h all sexes were determined using molecular sex determination according to Griffiths *et al*. (1998).

The following day (day 1) all chicks were cross-fostered. These final experimental nests contained two males (one control, one Flu chick) and two females (one control, one Flu chick) matched for (actual) mass, position in the laying sequence and hatching date. Subsequently, we assessed survival of every individual by recording the presence of each chick in the enclosures every day. To determine growth rates we measured body mass (to the nearest 0·5 g using a Pesola spring balance) at least every second or third day.

Table 1. Egg mass [(g), mean ± SE] and chick mass at the day of cross-fostering [(g), mean ± SE] separately for the four experimental groups

Treatment	Sex	Egg mass	Body mass at CF
Oil	Male	36·19 ± 0·73	29·82 ± 0·77
Oil	Female	36·64 ± 0·64	29·31 ± 0·66
Flutamide	Male	36·39 ± 0·66	29·27 ± 0·89
Flutamide	Female	36·95 ± 0·58	29·03 ± 0·90

Of the 351 eggs that were injected (C eggs: 84 oil, 83 Flu; B eggs: 88 oil, 96 Flu), 241 hatched. The total hatchability was 69·8% (of which 52% control chicks, 48% Flu chicks), similar to earlier injection studies (e.g. Eising *et al*. 2001). We were able to create 29 (19 B chicks, 10 C chicks) nests (116 chicks) in which all four chicks could be matched for mass, hatching date, laying order, sex and treatment. Chicks that could not be used in the experiment ($n = 125$) were allocated to foster nests, creating broods of three chicks that subsequently were no longer followed.

There were no significant differences in egg mass or chick body mass at cross-fostering between the experimental chicks of the different treatment/sex groups within a nest (Table 1, paired sample t-test, $P > 0·10$ in all cases).

Black-headed gull chicks are especially vulnerable to adverse weather conditions in the period between about 10–14 days of age, when cold and rainy weather can have a strong impact on the survival probability (e.g. Jennings & Soulsby 1958). During this vulnerable period, about 24 h of continuous rain occurred on the day 13 of the experiment. Because all chicks hatched within 4 days, nearly all chicks died due to chilling (19 survivors: seven Flu females, five oil females, five oil males, two Flu males). Therefore, the experiment was terminated. All subsequent analyses will be restricted to the early developmental period, when the chicks were a maximum of 10–13 days old, which is the period before the development of sexual size dimorphism.

IMMUNOCOMPETENCE

In order to investigate the cell-mediated immunity (CMI), we injected intradermally 0·04 mL of 1 mg/mL phytohaemagglutinin-P (PHA, Sigma©) dissolved in phosphate-buffered saline (PBS) into the ball of the foot 1 day after hatching (for details on the injection method see Müller *et al*. 2003). *In vivo* injection of PHA produces a local swelling due to a prominent perivescular accumulation of T-lymphocytes followed by macrophage infiltration and is considered to be a reliable method of measuring CMI (Smits, Bortolotti & Tella 1999). Three repeated measurements of the swelling were taken just prior to injection (initial), and

a further three 24 h (± 1 h) after injection (final). Because the repeatability of the successive measurements was high ($r = 0{\cdot}98$, $P < 0{\cdot}001$) we used the mean value of the three measurements for analysis. The difference between initial and final measurements was used as the response estimate, subsequently called CMI (Smits *et al.* 1999).

In total, we measured CMI in 101 of the 116 chicks, with the remaining 15 chicks dead before the final measurement. In 20 of the 29 nests we were able to measure all four chicks. Only these clutches were included in the analysis of CMI, as brood size very probably influences the nutritional circumstances and thus potentially the CMI (reviewed by Alonso-Alvarez & Tella 2001).

STATISTICAL ANALYSES

All body mass and CMI data were analysed using hierarchical linear models (Rasbash *et al.* 2000) to test the effect of sex and treatment in a nested design. The following variables were included in the models: treatment, sex, laying position of the egg and all possible interactions. In the case of body mass, number of siblings was also included, as well age and age^2 to model the growth curve (see Eising *et al.* 2001 for a similar approach). Because all nests started with a standard number of four chicks, the decrease in sibling number due to mortality correlated significantly with age. To avoid the problem of colinearity between both factors in the model, we calculated the residuals of the number of siblings on age and included these in the model.

Significance was tested using the Wald statistic, which follows a χ^2 distribution, and accepted at $P < 0{\cdot}05$ (two-tailed). Survival data were analysed using the Wilcoxon Gehan statistics in the Life-tables option in SPSS.

Results

BODY MASS

Body mass increased with age and age^2 [age: estimate: 4·527 (g/day), error: 0·604, Δdev 53·117, d.f. 1, $P < 0{\cdot}0001$; age^2: estimate: 0·351 (g/day^2), error: 0·043, Δdev 62·263, d.f. 1, $P < 0{\cdot}0001$]. The number of siblings in a nest, corrected for age, positively affected body mass (residual siblings: estimate: 2·332, error: 0·935, Δdev 6·14, d.f. 1, $P = 0{\cdot}01$). Treatment correlated with body mass in interaction with age and sex (treatment × age × sex: estimate: 1·171, error: 0·212, Δdev 29·777, d.f. 1, $P < 0{\cdot}0001$). Thus Flutamide treatment had a sex-specific effect on body mass gain.

In a subsequent *post hoc* analysis, sex turned out not to influence chick's body mass when the analysis was restricted to control chicks (sex × age: estimate: – 0·177, error: 0·172, Δdev 1·056, d.f. 1, $P = 0{\cdot}30$), while residual siblings, age and age^2 still contributed significantly to the explained variance. In contrast, within the Flutamide group, body mass gain was different according to sex (sex × age: estimate: 1·101 error: 0·173, Δdev 37·430, d.f. 1, $P < 0{\cdot}0001$), while residual siblings, age and age^2 again contributed significantly to the explained variance. Analysing the sexes separately showed that male body mass positively correlated with treatment, in interaction with age (treatment × age estimate: 1·009, error: 0·378, Δdev 7·028, d.f. 1, $P = 0{\cdot}008$), while in the same model also residual siblings, age and age^2 contributed significantly. In contrast Flutamide negatively affected female body mass (treatment × age estimate: –1·506, error: 0·309, Δdev 22·632, d.f. 1, $P < 0{\cdot}0001$), while in the same model residuals siblings, age and age^2 also contributed significantly (Fig. 1).

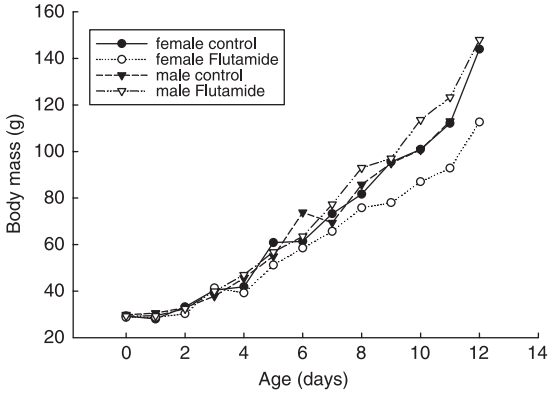

Fig. 1. Mean body mass (g) for female (filled circle Oil-females, open circle Flu-females) and male chicks (filled triangle Oil-males, open triangle Flu-males) in relation to age between hatching and day 10.

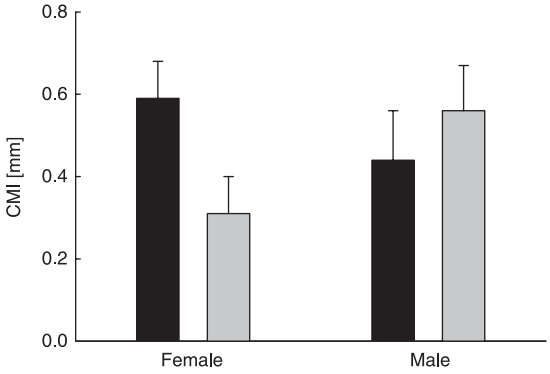

Fig. 2. Cell-mediated immunity [CMI (mm) ± SE] measured 2 days after hatching of female [oil females (black bar), Flu females (grey bar)] and male chicks [oil males (black bar), Flu males (grey bar)].

CELL-MEDIATED IMMUNITY

In the control situation the sexes did not differ in their CMI (sex: estimate: – 0·077, error: 0·072, Δdev 1·096, d.f. 1, $P = 0{\cdot}30$, Fig. 2), nor did laying order affect the CMI (laying order: estimate: 0·078, error: 0·122, Δdev 0. 405, d.f. 1, $P = 0{\cdot}52$). There was no effect of body mass at injection on CMI (body mass estimate: 0·018, error: 0·014, Δdev 1·567, d.f. 1, $P = 0{\cdot}21$). Flutamide treatment affected CMI in interaction with sex (treatment × sex: estimate 0·201, error: 0·099, Δdev

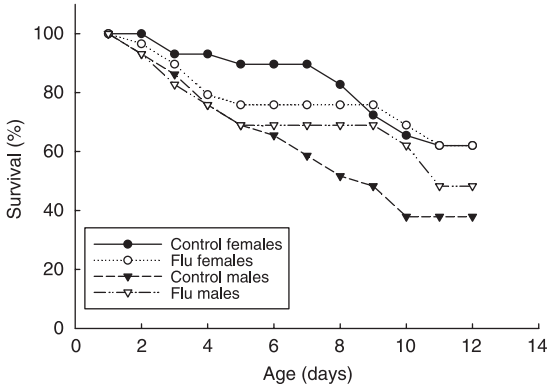

Fig. 3. Cumulative survival [%] of Oil-females (filled circle), Oil-males (filled triangle), Flu-females (open circle) and Flu-males (open triangle).

3·987, d.f. 1, $P = 0·045$, Fig. 2). There was no effect of treatment on CMI when analysing male chicks separately (treatment: estimate: 0·117, error: 0·159, Δdev 0·532, d.f. 1, $P = 0·47$, Fig. 2). However, Flutamide negatively affected female CMI (treatment: estimate: –0·285, error: 0·129, Δdev 4·599, d.f. 1, $P = 0·03$, Fig. 2). Within the Flu chicks, females tended to have a lower CMI compared to male chicks (sex: estimate: 0·124, error: 0·062, Δdev 3·62, d.f. 1, $P = 0·057$).

Individual CMI scores did not correlate with survival probabilities (estimate: –0·008, error: 0·106, Δdev 0·005, d.f. 1, $P = 0·94$).

SURVIVAL

When comparing the two sexes within the control chicks, survival was different for the sexes with males suffering higher mortality (Wilcoxon Gehan statistic = 6·48, $P = 0·01$; 37·9% survival in control males compared to 62·1% in control females, Fig. 3). In contrast, Flu chicks did not show a sex-specific difference in their survival (Wilcoxon Gehan statistic = 0·803, $P = 0·37$; 48·3% survival in Flu males compared to 62·1% Flu female survival, Fig. 3). Treatment did not significantly affect survival probabilities either in males (Wilcoxon Gehan statistic = 0·769, $P = 0·38$) or in females (Wilcoxon Gehan statistic = 0·191, $P = 0·66$). Given these results, we expect a significant interaction effect between treatment and sex (as in case of the CMI). However, a statistically test for interaction effects using the life-tables approach is not yet possible.

Discussion

MALE BIASED MORTALITY IN THE CONTROL CHICKS

This study shows that male black-headed gull chicks are at a survival disadvantage when compared to females in a situation where we controlled for age and size differences between the siblings and where competition was enhanced by brood size enlargement (Fig. 2). This is consistent with earlier studies, showing that the larger sex in sexually size-dimorphic species is more vulnerable during the nestling phase when food is limited (Røskraft & Slagsvold 1985; Nager *et al*. 2000). While black-headed gulls are sexually dimorphic, we found this difference in survival probability already during the first 2 weeks post-hatching where no sex differences in the food demand are expected, as there is neither a sex difference in the metabolic rate (Eising *et al*. 2003) nor in growth (Fig. 1). This renders it unlikely that differences in energetic requirements are responsible for this sex-specific mortality pattern and points to other features of the male phenotype such as a reduced immunocompetence (e.g. Olsen & Kovacs 1996; Klein 2000). We did not find evidence that the difference in survival probability between the sexes as found in this study depended on the cell-mediated immunity (CMI). The on-average somewhat lower CMI in control males did not differ statistically from control females (Fig. 3). Thus, we did not find support for earlier evidence for sex differences in CMI (Müller *et al*. 2003; see also Fargallo *et al*. 2002; Tschirren *et al*. 2003) or the decrease in CMI with laying position (Müller *et al*. 2003). Both have been suggested to be related to the production of endogenous testosterone in males and the increase of maternal yolk androgens with laying sequence (Eising *et al*. 2001).

While chicks appeared to have no sexual dimorphism in their growth pattern or CMI, the sexes may be dimorphic in their constitution at hatching. For the closely related lesser black-backed gulls (*Larus fuscus*) it has been shown that male hatchlings are skeletally larger but not heavier than females (Nager *et al*. 1999; Verboven *et al*. 2003). This is consistent with data of the sexually dimorphic domestic chicken (Nielsen & Torday 1985; Henry & Burke 1998), showing that male and female embryos differ in the prenatal allocation pattern of nutritional factors. These different allocation strategies potentially influence the amount of yolk reserves male and female chicks carry post-hatching as well as structural size differences, with sex-specific impacts on their early survival and their vulnerability to food shortage early in life (reviewed in Williams 1994).

FLUTAMIDE EFFECTS IN FEMALES

Flutamide negatively affected body mass gain in females and reduced their CMI. This may be due to the fact that Flutamide induced detrimental effects in female chicks by blocking the beneficial effects of maternal testosterone. Maternal androgens are known to be present in substantial amounts in gull eggs (e.g. Eising *et al*. 2001) and embryonic exposure to maternal testosterone is known to influence competitive skills and growth (Schwabl 1993, 1996; Eising *et al*. 2001). The level of sibling competition will have been high in the experimentally enlarged broods. Flutamide would also have blocked the exposure to endogenous testosterone; however, this is not likely to modify the process of

feminization, which is dependent mainly on oestrogen (Balthazart & Adkins-Regan 2003). Although we expected a positive effect of Flutamide on CMI, such a direct effect may have been masked by a negative effect of Flutamide on competitive abilities and thus nutritional state, as CMI has been shown to correlate with body condition (Alonso-Alvarez & Tella 2001). Hence this may have affected the CMI at a stage where effects on body mass were not yet detectable.

FLUTAMIDE EFFECTS IN MALES

The positive effects of experimental *in-ovo* treatment with Flutamide in males are consistent with our hypothesis that the ratio of testosterone to oestrogen is involved in the expression of the male phenotype disadvantage. Flu males benefited from a reduced testosterone exposure, particularly in terms of enhanced body mass gain. The effects on CMI and survival were in the same direction, but less strong than expected, and did not reach statistical significance. Perhaps Flutamide affected the embryonic allocation pattern in males, possibly 'feminizing' their structural size and yolk reserves at hatching.

The small effect size in males may relate to the fact that Flutamide not only blocks the exposure to endogenously produced testosterone, but also the beneficial exposure to maternal androgens (e.g. Schwabl 1996; Eising *et al*. 2001). Thus, the results of this study are very probably an underestimation of the contribution of endogenous testosterone to the male phenotype disadvantage. Maternal and endogenous androgens may have opposite effects on male performance due to the different periods when these hormones are present or to differences in their concentration (e.g. domestic chicken: Woods *et al*. 1975; Müller *et al*. 2002).

Alternatively, if Flutamide mainly prevented the action of maternal testosterone, the opposing effects of a reduced testosterone exposure in male and female chicks might indicate sex-specific effects of maternal testosterone (Strasser & Schwabl 2004). Antagonistic effects on male and female offspring may create an upper threshold of yolk androgen deposition due to a trade-off between beneficial effects on one sex and detrimental effects on the other sex, favouring intermediate concentrations.

Finally, the treatment with Flutamide could have led to an increase of plasma testosterone via blockade of negative feedback on luteinizing hormone, as has been shown in implantation experiments with adult birds (Schwabl & Kriner 1991; Soma, Sullivan & Wingfield 1999). This could represent a possible pathway as the hypothalamo–pituitary axis already becomes functional at days 12–14 of embryonic development (Li, Alston-Mills & Ottinger 1991; Ottinger & Abelnabi 1997). The high levels of plasma testosterone might then induce demasculinization, as has been shown for the administration of exogenous testosterone before the end of the critical period (Wilson & Glick 1970; Sayag *et al*. 1989). Such a de-masculinization may come about by the fact that the enhanced levels of plasma testosterone provide additional substrate for aromatase activity and hence might be transferred into oestrogen (Balthazart & Adkins-Regan 2003). Independently of the exact pathway, male chicks clearly benefited from a reduction in testosterone exposure.

In summary, the male phenotype disadvantage is not linked exclusively to differences in the energetic requirements between the sexes when developing sexual size dimorphism as it is already present just after hatching. Male chicks benefited from Flutamide treatment. This supports the idea that sex differences in endocrine state are involved in the expression of the male disadvantage. Female chicks hatching from Flutamide treated eggs were disadvantaged, which may relate to blocking of the positive effects of embryonic exposure to maternal androgens on competitive skills.

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