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Differential effects of yolk hormones on maternal and paternal contribution to parental care

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In species with biparental care, a female gains fitness benefits from the joint reproductive investment of herself and her partner, but pays only the costs of her own care. Selection thus favours mechanisms that allow females to elicit a higher paternal investment from her partner. In oviparous species, the allocation of maternal yolk androgens into the eggs might represent such a female adaptation to sexually antagonistic selection. To test this hypothesis, we experimentally blocked the effects of maternal yolk androgens by an injection of the anti-androgen flutamide or a control substance in the eggs of great tits (Parus major). After hatching, we subsequently manipulated the food demand of the brood in a brood size manipulation experiment, and recorded the parental feeding rates. We found that the males’ food provisioning rates were not significantly influenced by the actions of maternal yolk androgens, while females adjusted their parental effort to androgen-mediated nestling signals, in particular in enlarged broods. These results show that female great tits do not exploit the male’s contribution to parental care by allocating high concentrations of yolk androgens into their eggs. It however indicates, that variation in yolk androgen allocation among females has evolved through a process of coadaptation that matches maternal food provisioning and offspring demand.

**Keywords:** coadaptation, food provisioning, great tit, maternal effects, maternal yolk androgens, parental investment, parent-offspring communication, Parus major, sexual conflict
In sexually reproducing species, a conflict over parental investment occurs because each parent gains fitness benefits from the joint reproductive investment of both partners, but pays only the costs of its own care. Selection will thus favour adaptations that successfully manipulate the genetically unrelated partner into elevating its parental investment, which, as a consequence, will allow the manipulator to reduce its own reproductive contribution (Trivers 1972; Houston & Davies 1985; Lessells 1999; McNamara et al. 1999; Arnqvist & Rowe 2005; Houston et al. 2005).

In blue tits (*Cyanistes caeruleus*), for example, a sexual conflict over the degree of hatching asynchrony occurs (Slagsvold et al. 1994, 1995). Females pay relatively lower costs of parental care in terms of future survival when raising even-aged nestlings, while males survive better when caring for an asynchronous brood (Slagsvold et al. 1994, 1995). While the total cost of parental care is similar for synchronous and asynchronous broods, the different hatching patterns thus alter the relative reproductive burden on the two sexes (Slagsvold et al. 1994, 1995). Because only the female incubates in this species, she can control the degree of hatching asynchrony by varying the start of incubation, and is thus likely to win this conflict (Slagsvold et al. 1994, 1995).

Not only the start of incubation, but any reproductive behaviour that is under female control alone, has the potential to mediate sexual conflicts over reproductive decisions in favour of the female’s own interest. In birds (Schwabl 1993; Groothuis et al. 2005), fish (McCormick 1999), and reptiles (Bowden et al. 2000; Lovern & Wade 2003), for example, females are known to deposit androgens and other hormones into their eggs. The amount of yolk
hormones varies however greatly, not only among species, but also within and among clutches of the same species and even among clutches of the same female. Variation within clutches, i.e. increasing or decreasing hormone concentrations with laying order, might aim at mitigating the competitive asynchrony among siblings caused by asynchronous hatching (e.g. Eising et al. 2001; Pilz et al. 2003) or enhance these effects to facilitate brood reduction when food is limited (Schwabl et al. 1997). However, although variation in the amount of yolk hormones deposited among clutches of different females is typically even larger (e.g. Reed & Vleck 2001; Groothuis & Schwabl 2002; Pilz et al. 2003; Tschirren et al. 2004), the mechanisms driving this variation are as yet poorly understood (Groothuis et al. 2005, Müller et al. 2007b).

Experimental studies on a number of bird species have shown that yolk androgens deposited by the mother can have profound effects on the development, morphology and food acquisition behaviour of nestlings (reviewed in Groothuis et al. 2005). Young birds originating from eggs with experimentally high yolk androgens concentrations showed, for example, a higher begging effort (Schwabl, 1996; Eising & Groothuis 2003; von Engelhardt et al. 2006; but see Pilz et al. 2004; Boncoraglio et al. 2006) and a higher growth rate (Schwabl, 1996; Eising et al. 2001; Tschirren et al. 2005) than their unmanipulated siblings. Further, maternal yolk androgens can promote the development of the musculus complexus, a neck muscle involved in food acquisition (Lipar & Ketterson 2000), and beak flange size, which might stimulate parental food provisioning (Müller et al. 2007b). Yolk hormones can thus influence a variety of physiological, behavioural and
morphological nestling traits, all of which may affect parental feeding decisions.

If males are especially sensitive to such hormone-dependent nestling signals, then the deposition of yolk androgens by the female could represent a sexually antagonistic adaptation that aims at exploiting the male's contribution to parental care, as recently suggested by Michl et al. (2005), Moreno-Rueda (2007) and Müller et al. (2007a). To test this, we experimentally inhibited the effects of maternal yolk androgens by injecting flutamide, an androgen receptor inhibitor, or a control substance into the eggs of free-living great tits (Parus major), and filmed the food provisioning behaviour of the parents to measure their investment.

Flutamide directly competes with testosterone and testosterone-metabolites for binding to androgen receptors (Simard et al. 1986), and previous work on chicken (Gallus gallus; Burke 1996), red-winged blackbirds (Lipar & Ketterson 2000) and black-headed gulls (Müller et al. 2005) has confirmed its anti-androgenic actions when injected in ovo. If the deposition of yolk androgens into the eggs is a sexually antagonistic adaptation of the female to increase the male's contribution to parental care, we predict 1) higher feeding rates of the male in control-injected compared to flutamide-injected (i.e. androgen-blocked) broods, and 2) that the effect of yolk androgens on the parental food provisioning behaviour will be most pronounced when the nestlings' degree of hunger, and thereby the cost of rearing the brood, is increased (i.e. in experimentally enlarged broods).
METHODS

Study Site, Species and Flutamide Injection

The experiment was performed in 2003 in a great tit population (*Parus major*) breeding in nest boxes in a forest near Bern, Switzerland (“Forst”, 46°54’N 7°17’E / 46°57’N 7°21’E). Great tits are socially monogamous passerines that rear one, or in a few cases two broods per year. Only females build the nest and incubate the eggs but both parents feed the young during the nestling stage (see e.g. Tschirren et al. 2005a for a typical division of work between the sexes).

All nest boxes were cleaned before the start of the breeding season (February) to remove nest-based ectoparasites, which can influence yolk androgen deposition (Tschirren et al. 2004). From the beginning of the breeding season (April) onwards, we regularly visited nest boxes to determine the start of nest building and egg laying. After clutch completion, we injected all eggs of a clutch with either 0.0319 µmol of the anti-androgen Flutamide (Fluka, Switzerland) dissolved in 5 µl ethanol (70%) or with 5 µl ethanol (70%) as a control (see Lipar & Ketterson 2000 for details). The injected dose of flutamide was based on the amounts used in Burke (1996) and Lipar & Ketterson (2000), adjusted for an average yolk mass of 352 mg in our study population. The injections were done in the field using a 25 µl syringe (Hamilton 702LT) and a 25 G needle (see Tschirren et al. 2005b for details).

During the injection procedure, the eggs were illuminated from beneath using a cold light source (Intralux 4000, Volpi, Switzerland) to ensure that the tip of the needle penetrated the yolk membrane. The hole in the eggshell was sealed by applying a small drop of tissue adhesive (Vetseal, B. Braun).
Medical, Switzerland). The average hatching success of the eggs was 77.2%. It did not significantly differ between flutamide-injected and control eggs ($\chi^2=0.45$, $P=0.502$, N=534).

**Brood Size Manipulation**

One day after hatching, we created broods of reduced (-2 nestlings compared to original clutch size) and enlarged size (+2 nestlings compared to original clutch size) by partially exchanging randomly chosen nestlings among nests with the same flutamide treatment, the same hatching date and a similar clutch size. After the manipulation, both enlarged and reduced nests contained own and foster nestlings of the same flutamide-treatment group, and the manipulated brood sizes remained within the range of natural variation observed in our study population (5–12 nestlings / brood). This manipulation allowed us to assess the investment of the parents in response to an elevated or reduced food demand of the brood, and the interaction effects between the food demand of the brood and the effects of maternal yolk androgens (i.e. a 2x2 design).

The original clutch size did not differ significantly between treatment groups (brood size manipulation, $F_{1, 46} = 0.01$, $P = 0.941$; flutamide treatment, $F_{1, 46} = 0.81$, $p = 0.373$; interaction, $F_{1, 45} = 0.07$, $P = 0.800$). After the manipulation, the brood size was significantly larger in enlarged compared to reduced broods (brood size manipulation, $F_{1, 46} = 187.24$, $P < 0.001$; $R^2=0.80$), but did not differ significantly between the flutamide-treatment groups ($F_{1, 46} = 0.42$, $P = 0.520$; interaction, $F_{1, 45} = 0.08$, $P = 0.776$).
Parental Food Provisioning

Ten days post-hatching, when the nestlings’ food demand is highest (Gebhardt-Henrich 1990; Keller & Van Noordwijk 1994), we installed a video camera sensitive to infrared and equipped with an infrared lamp in the nest box to assess the parental investment in response to the flutamide treatment and the brood size manipulation. Video recording started 30 minutes after camera installation and the food provisioning behaviour of the parents was filmed during the following 100 minutes. After the filming, nestlings were weighted and ringed with individually numbered aluminium rings.

A total of 49 broods (11 enlarged, control-injected broods, 14 reduced, control-injected broods, 11 enlarged, flutamide-injected broods, and 13 reduced, flutamide-injected broods) were filmed. The analysis of the recordings was performed blindly with respect to the treatment of the nest. Males and females can be visually identified on the videos. Only visits during which the male or female parent delivered food to at least one nestling were counted as a feeding visit. In addition to the number of parental feeding visits, we also classified the prey size brought to the nestlings as small, intermediate or large (Kölliker et al. 1998). The food quantity delivered to the nestlings was then calculated as the product of feedings per hour and mean prey size.

Because the analyses of feeding visits and food quantity delivered to the brood were qualitatively very similar (see also Moreno et al. 1995; Neuenschwander et al. 2003) only the results of the former are presented here.

The disturbance of the nest during the installation of the camera (performed within < 5 minutes) and the filming within the nest boxes (method
established in previous years; see e.g. Tschirren et al. 2005a) did not have a negative effect on the feeding behaviour of the parents. Both parents fed the nestlings on a regular basis during the filming in all experimental nests, and no nest desertion or nestling mortality was observed during the filming. The video recording or the disturbance of the nests during the manipulations did not have any observable adverse short- or long-term effects on the nestlings, and their weight (mean: 15.77 ± 0.10g) was similar to the weight of great tit nestlings in our study population in previous years (range of mean weights between 1997 and 2002: 14.49g–16.21g, depending on food availability).

**Statistical Analyses**

The flutamide treatment and the brood size manipulation influenced the feeding behaviour of males and females differently (see Results). We thus analysed the feeding behaviour of mothers and fathers separately. Maternal and paternal feeding visits were analysed with ANCOVAs including the flutamide treatment, the brood size manipulation, and their interaction as fixed effects and nestling body mass at filming as a covariate. The interaction was removed from the final model if non-significant. For the analysis of differences between flutamide-injected and control-injected broods within a given brood size manipulation group we used least-square mean contrasts. All tests were two-tailed with a significance level of $P \leq 0.05$. Residuals of the models were tested for normality using Shapiro-Wilk tests and homoscedasticity using Bartlett tests to ensure that the assumptions for parametric testing were fulfilled. Means ± 1 S.E. are presented in the results.
and figures. Statistical analyses were performed using JMP IN 5.1 (Sall & Lehmann 1996).

RESULTS

The flutamide treatment and the brood size manipulation influenced the feeding behaviour of the two parents differently (three-way interaction: parental sex x brood size manipulation x flutamide treatment, $F_{1, 44}= 6.08$, $P= 0.018$). We therefore analysed the feeding behaviour of mothers and fathers separately.

Paternal Food Provisioning

Contrary to our prediction, we found no significant effect of the flutamide treatment ($F_{1, 45} = 0.17$, $P = 0.685$) and no significant interaction effect of the flutamide treatment and the brood size manipulation ($F_{1, 44} < 0.01$, $P = 0.993$) on the males’ feeding rates (Fig. 1a). Independent of the flutamide treatment of the young, males raising an enlarged brood fed at significantly higher rates than males raising a reduced brood ($F_{1, 45} = 4.87$, $P = 0.033$, Fig. 1a).

Male feeding rates were not significantly influenced by nestling body mass at filming ($F_{1, 45}= 2.61$, $P= 0.114$). Further, no effect of the brood size manipulation on the males’ feeding visits per nestling were found ($F_{1, 45} = 1.95$, $P = 0.170$), showing that males increased their feeding effort in enlarged broods to keep the food provisioning per nestling constant. Male feeding rates were not significantly influenced by the date of filming ($F_{1, 41}= 2.19$, $P= 0.147$), the time of filming ($F_{1, 41}< 0.01$, $P= 0.932$), the original clutch size ($F_{1, 41}= 0.31$, $P=0.578$), or the partner’s feeding rate ($F_{1, 41}= 0.174$, $P=0.679$).
Maternal Food Provisioning

We found a significant interaction effect of the flutamide treatment and the brood size manipulation on the mother’s feeding visits ($F_{1, 44} = 8.65, P = 0.005$, Fig. 1b). When the food demand of the brood was low (i.e. in reduced broods), mothers did not feed control-injected and flutamide-injected nestlings at significantly different rates (contrast: $F_{1, 44} = 0.04, P = 0.851$), whereas when the food demand of the brood was high (i.e. in enlarged broods), mothers of control-injected broods showed significantly higher feeding rates compared to mothers of flutamide-injected broods (contrast: $F_{1, 44} = 17.15, P <0.001$). Similarly, within the control-injected group, mothers feeding an enlarged brood showed significantly higher feeding rates than mothers feeding a reduced brood (contrast $F_{1, 44} = 21.50, P <0.001$), while in the flutamide-injected group the difference in the females' food provisioning behaviour between enlarged and reduced broods was not statistically significant (contrast $F_{1, 44} = 0.46, P =0.499$). Thus, females did not adjust their food provisioning to the higher food demand of enlarged broods when the actions of maternal yolk hormones were blocked. Similar results were found when analysing the females’ feeding visits per nestling instead of the total feeding visits per brood (interaction brood size manipulation x hormone treatment: $F_{1, 44} = 4.30, P =0.044$), indicating that females overcompensated in response to the brood size manipulation.

Female feeding rates significantly increased during the breeding season ($F_{1, 43} = 4.27, P =0.045$) and tended to decrease with increasing nestling body mass ($F_{1, 43} = 3.03, P =0.089$). However, including these covariates into the analysis did not change the interaction effect between the
brood size manipulation and the hormone treatment on female feeding rates (interaction: $F_{1,43}= 10.28, P= 0.003$). Female feeding rates were not significantly influenced by the time of video recording ($F_{1,40}= 0.13, P= 0.723$), the original clutch size ($F_{1,40}= 0.17, P=0.681$), or the partner's feeding rate ($F_{1,40}= 0.13, P=0.718$).

**DISCUSSION**

In this study we experimentally investigated if maternal yolk androgens represent a mechanism by which females can manipulate their partner's investment in parental care, as recently suggested by Michl et al. (2005), Moreno-Rueda (2007) and Müller et al. (2007a). Counter to the predictions of this hypothesis, we found that males adjusted their feeding effort solely to the number of nestlings present in the nest, irrespective of the flutamide-treatment of the young. Likewise, in control-injected broods, females increased their feeding visits when caring for an enlarged brood. However, when the actions of maternal yolk androgens were experimentally inhibited, (i.e. in the flutamide-injected group), an increased food demand of the brood did not lead to a higher maternal investment. Thereby these results indicate that yolk androgens allow nestlings to elicit higher feeding rates from their mother, especially when their food demand is increased (i.e. when environmental conditions get unfavourable; see also Pilz et al. 2004).

Our finding that yolk hormones have an effect on maternal, but not on paternal investment, suggests that selection may have reduced the males' susceptibility to yolk androgen-mediated nestling signals to resist the exploitation of their parental effort by the female (see also Müller et al. 2007a).
Indeed, evidence that males ignore certain nestling displays has been found in canaries, where males adjust their food provisioning to the chicks' begging posture only, while females respond to a variety of offspring signals (Kilner 2002b). Similarly, male great tits adjust their feeding behaviour to visual displays, while females integrate visual as well as vocal nestling signals (Kilner 2002a).

While males were not susceptible to the effects of yolk androgens, females did adjust their feeding behaviour in response to the flutamide treatment, especially when feeding an enlarged brood. This shows that maternal yolk hormones play an important role in regulating mother-offspring interactions. Indeed, Kölliker et al. (2000) showed by means of a partial cross-fostering experiment, that the level of food solicitation by the nestlings is largely dependent on their nest of origin, and that the female’s increase in food provisioning in response to an increased demand was positively correlated to the natural levels of begging intensity of her offspring. No such correlation was found in the paternal line. Although the role of (additive) genetic variation cannot be excluded (Kölliker et al. 2000), our finding supports the idea that this correlation between offspring demand and maternal response may at least partly be mediated or enhanced by maternal effects (see also Kölliker et al. 2000; Kölliker et al. 2005).

The goal of this study was to investigate the effects of yolk hormones on parental feeding investment, which does not require a direct measurement of the numerous potential pathways that could mediate such effects. However, now we know that yolk hormones do indeed affect maternal investment, and that this effect is independent of nestling body mass, elucidating these
proximate mechanisms is an obvious next step. In particular, to test whether
males specifically ignore yolk androgen-dependent nestling signals to avoid
exploitation of parental care by the female, we will need to experimentally
investigate the effects of yolk hormones on as many different aspects of
offspring behaviour and morphology as possible, and to test for sex-specific
sensitivities to these traits.

In conclusion, our study shows that female great tits do not exploit the
male’s contribution to parental care by differentially allocating yolk androgens
into the eggs. However, the females’ own adjustment of parental effort in
response to yolk androgen-mediated nestling signals indicates that maternal
hormones do play an important role in mother-offspring communication, and
that variation in the deposition of yolk androgens between females may have
evolved to optimally match maternal supply and offspring demand, as
predicted by Kölliker et al. (2005). This maternal sensitivity to the actions of
yolk hormones might ensure a prime start for offspring, and particularly for
those of high reproductive value (see e.g. Gil et al. 1999, 2004; Tanvez et al.
2004; Loyau et al. 2007), even if environmental conditions get unfavourable.
Interestingly, the mother’s sensitivity to her offspring’s need also makes her
vulnerable to exploitation by her nestlings (Trivers 1974; Godfray 1995).
However, our finding that females did, unlike males, maintain their sensitivity
to yolk androgen-mediated nestling signals suggests that the costs of ignoring
such signals are sex-specific, and that in females they do not exceed the
benefits. Although more research is necessary to gain a full understanding of
the adaptive value of variation in yolk hormone deposition among females,
and the covariance between provisioning and soliciting behaviour caused by
genetic variation in maternal hormone levels, our study indicates that maternal
yolk hormones can play an important role in shaping the coadaptation among
mothers and their young.

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Figure 1

Feeding visits per hour of a) male, and b) female great tits in relation to the
flutamide treatment of the nestlings (nestlings originating from flutamide- or
control-injected eggs) and the manipulated brood size (feeding an enlarged or
reduced brood). Least square means + 1SE are shown.
**Fig. 1**

(a) Male feeding rate with control and Flutamide treatments.

(b) Female feeding rate with reduced and enlarged conditions.