Differential Maternal Testosterone Allocation among Siblings Benefits Both Mother and Offspring in the Zebra Finch *Taeniopygia guttata*

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Submitted July 24, 2010; Accepted March 11, 2011; Electronically published June 1, 2011

Dryad data: http://dx.doi.org/10.5061/dryad.8809.

abstract: Parents are selected to preferentially invest in the offspring with highest reproductive value. One mechanism for achieving this is the modification of competitive asymmetries between siblings by maternal hormones. In many organisms, offspring value varies according to birth position in the brood, which determines survival chances and competitive advantage over access to resources. In birds, variation in yolk androgen allocation over the laying sequence is thought to modulate dominance of senior chicks over junior brood mates. We tested this hypothesis in zebra finches, which show a naturally decreasing pattern of within-clutch testosterone allocation. We abolished these within-clutch differences by experimentally elevating yolk testosterone levels in eggs 2–6 to the level of egg 1, and we assessed fitness measures for junior offspring (eggs 2–6), senior offspring (egg 1), and their mothers. Testosterone-injected eggs hatched later than control eggs. Junior, but not senior, chicks in testosterone-treated broods attained poorer phenotypic quality compared to control broods, which was not compensated for by positive effects on seniors. Mothers were generally unaffected by clutch treatment. Thus, naturally decreasing within-clutch yolk testosterone allocation appears to benefit all family members and does not generally enhance brood reduction by favoring senior chicks, in contrast to the widely held assumption.

Keywords: maternal effects, sibling competition, hatching time, body mass, chick survival, birds.

Introduction

In species where several offspring are produced per breeding event, initial brood sizes are generally larger than those surviving the period of parental care because environmental conditions and offspring viability are not entirely predictable (Stearns 1992; Mock and Parker 1997). Parental allocation is therefore expected to be adaptively adjusted to asymmetries in the reproductive value of different offspring, in order to maximize the fitness return to parents (Godfray 1995; Mock and Parker 1997). This is thought to result in preferential investment in "core" offspring under normal conditions, allowing "marginal" offspring to survive only when environmental conditions are exceptionally favorable or if other offspring fail to thrive (Mock and Forbes 1995; Mock and Parker 1997). In addition, in sexually reproducing organisms, parents and offspring share only half their genome (Hamilton 1964). From vertebrates to plants, the genetic parent-offspring conflict that results should select for mechanisms by which parents adjust resource distribution according to offspring quality, especially when direct competition among siblings strongly affects the viability of the progeny (Trivers 1974; Roach and Wulff 1987; Mock 2004; Hudson and Trillmich 2008). Thus, important questions are to what extent differential distribution of resources to individual offspring promotes parental and/or offspring fitness and how this factor affects sibling conflicts.

Parents may bias their resources toward specific offspring not only postnatally, for example, by preferential feeding, but also prenatally, during the embryonic development. A plethora of studies have analyzed the effects of signals produced by offspring to manipulate parental investment, especially in species with posthatching parental care and direct competition among offspring (e.g., Mock and Parker 1997; Wright and Leonard 2002). However, the reverse process, that is, the role of information provided by parents to offspring on the parental optimum and consequent allocation strategy, has been studied much less (Kilner and Hinde 2008; Hinde et al. 2010). Information

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Am. Nat. 2011. Vol. 178, pp. 64–74.
DOI: 10.1086/660278

flow from parents to offspring has been observed across all taxa studied so far, and it can be mediated, among other ways, by differential transfer of hormones produced by mothers to the eggs or developing offspring (Ravishankar et al. 1995; Mousseau and Fox 1998; Groothuis et al. 2005; Gil 2008; see also above).

Birds are excellent organisms for studying how mothers may manipulate sibling competition by prenatal hormone-mediated maternal effects. In most bird species, offspring hatch sequentially, resulting in an advantage for first-hatching young in competing over limited resources provided by parents. Mothers may regulate the extent of age- and size-mediated competitive asymmetries at hatching via differential egg provisioning (Bernardo 1996; Groothuis et al. 2005). Indeed, systematic variation over the laying sequence in size and composition of avian eggs has been thought to reflect a strategy to either enhance or reduce the competitive asymmetry between senior chicks (first-hatched) and junior chicks (last-hatched; Stoleson and Beissinger 1995; Groothuis et al. 2005; Gil 2008).

Yolk testosterone of maternal origin modulates multiple chick traits relevant for sibling competition. It affects hatching time, either accelerating (Eising et al. 2001; Eising and Groothuis 2003) or delaying (Sockman and Schwabl 2000; von Engelhardt et al. 2006) incubation time, and enhances early survival (Eising and Groothuis 2003; von Engelhardt et al. 2006; but see Sockman and Schwabl 2000), early competitiveness (Schwabl 1996; Eising et al. 2001; Boncoraglio et al. 2006; von Engelhardt et al. 2006; Müller et al. 2008), and growth of the chicks (Schwabl 1996; Eising et al. 2001; von Engelhardt et al. 2006), with potential long-lasting effects until adulthood (Strasser and Schwabl 2004; Müller et al. 2008, 2009). Accordingly, an increase of testosterone concentration over the laying sequence is thought to counteract the disadvantage in sibling competition of the last-hatched chicks by enhancing their overall competitiveness (Schwabl 1993; Groothuis et al. 2005), while a decreasing pattern is thought to favor senior chicks at the cost of juniors, facilitating brood reduction (e.g., Schwabl et al. 1997). In addition, different patterns of within-clutch yolk androgen allocation may have certain costs and benefits for mothers too, which has been neglected in previous studies that have focused almost exclusively on the consequences for offspring only. Therefore, from an inclusive fitness perspective (Hamilton 1964), in order to assess the consequences of the allocation pattern adopted by the mothers with respect to these family conflicts, not only the effects on the treated offspring but also the effects on their untreated siblings and on the mother herself should be measured (Müller et al. 2007). To date, experimental testing of the effect of the within-clutch allocation of maternal androgens in relation to hatching asynchrony has been conducted in only one study on gulls (Eising et al. 2001), which showed a benefit of the natural increase of testosterone for the last-hatched chick at the cost of the first one, relative to the situation in which all eggs had similar androgen concentrations. However, such a test in a species that shows a decrease instead of an increase in yolk testosterone concentrations over the laying sequence, to the best of our knowledge has not yet been conducted. Moreover, previous egg injection studies assessed consequences only for the offspring and not for the mother, who is actually in charge of these maternal effects and may herself benefit or suffer from the effects of maternal androgens on her progeny.

Our study tested the effects of a manipulation of the natural yolk testosterone allocation pattern occurring over the laying sequence in the zebra finch *Taeniopygia guttata* by considering fitness measures for the mothers and for senior and junior offspring. The zebra finch is an especially intriguing model for this, since it is one of the few species in which yolk testosterone (hereafter, T) concentration decreases with the laying order in both captive (e.g., Gil et al. 1999; Rutstein et al. 2005; this study) and wild populations (S. C. Griffith, personal communication, 2011). It is also one of the few species in which effects of yolk T on chick growth and begging behavior have already been tested, although only at the level of between-brood variation (von Engelhardt et al. 2006). In particular, experimentally increased yolk T levels in the whole brood resulted in enhanced early competitiveness and growth of female chicks (von Engelhardt et al. 2006), suggesting that the decreasing pattern may facilitate brood reduction by favoring chicks of first-laid eggs. However, the same study also demonstrated that yolk T delays the time until hatching (von Engelhardt et al. 2006), an effect also observed in kestrels (Sockman and Schwabl 2000). The latter suggests that decreasing T allocation over the laying sequence might favor brood survival by postponing hatching of senior chicks and therefore may reduce the disadvantage of late hatching of chicks from last-laid eggs.

To test the function of the decrease of T concentrations with increasing laying order, we created two different types of clutches: control clutches in which yolk T levels followed the normal decreasing pattern over the laying sequence and experimental clutches in which T levels were the same in all eggs. This was done by raising T concentrations in all but the first egg of the clutch to the level of that first egg (see Sockman and Schwabl 2000 for a complementary approach on a different animal model). Maternal and offspring fitness estimates were assessed in the absence of fathers to prevent any possible confounding effect of postnatal sexual conflict on family members (Royle et al. 2002; Müller et al. 2007). Also, this choice allowed us to test for the effects of our treatment while removing the indirect effect potentially exerted by the mothers on the offspring

via yolk T-dependent manipulation of paternal care (Moreno-Rueda 2007; Müller et al. 2007). In order to create broods that were manageable for the mother only, brood sizes were set by day 4 (initial brood size) to about half the number of chicks in natural broods, that is, either two or three (one senior hatchling from egg 1 and one or two junior hatchlings from eggs 2–6). Broods of two can be raised by females alone without additional effort (Royle et al. 2002), whereas a brood size of three should reflect a more challenging condition for the mother but always within the natural range of workload experienced by a single parent in this species. Conversely, larger brood sizes would have subjected temporarily widowed mothers to excessive, unnatural stress condition during the rearing period. We evaluated the consequences of our T treatment by measuring hatching time, posthatching growth, and survival until fledging of senior and junior chicks. We took sex of the offspring into account when sample size allowed, since there is some evidence that yolk T concentrations may be sex specific under some circumstances (Gilbert et al. 2005; Rutstein et al. 2005) or may have sex-specific effects (von Engelhardt et al. 2006). To assess fitness consequences for the mothers, we measured their change in body mass during rearing, feeding rate at the peak of workload, response to an immunological challenge during the first breeding event, and effects on the subsequent breeding event.

Our predictions were these: (1) If the within-clutch testosterone allocation pattern maximizes maternal fitness, mothers should attain, irrespective of maternal strategy (i.e., either brood reduction or brood survival), larger benefits in control compared to those in T-treated nests, as measured by enhanced offspring phenotypic quality at independence, higher maternal phenotypic quality, and/or superior reproductive output during the subsequent breeding event. (2) If the decreasing pattern of within-clutch T allocation reflects a brood reduction strategy by mothers, senior offspring should obtain larger direct fitness benefits, as measured by higher phenotypic quality at independence, in control compared to those in T-treated nests, while the opposite is expected for their junior siblings. (3) If the decreasing pattern of T allocation reflects a brood survival strategy by mothers, junior offspring should attain higher phenotypic quality in control compared to that in T-treated nests, while the opposite is expected for their senior siblings.

Methods

Experimental Design

First, average concentration of yolk T for each position of the egg in the laying sequence was measured for clutches produced by birds of the same population that we used for the experiment. These data were used to determine the dosage of treatment. We then randomly assigned clutches to either T or control (hereafter, C) treatment; C clutches showed the natural decrease in T concentrations over the laying order and were injected with vehicle only, while T clutches were manipulated by elevating yolk T concentrations in eggs 2–6 to compensate for the natural decrease in yolk T concentration over the laying sequence, in such a way that, on average, each egg in the T clutch would have the same T concentration (fig. 1). First eggs were not injected in either the C or T clutches, since this was not necessary for comparing effects on senior chicks between T and C broods, and it increased sample sizes by increasing hatching success of senior eggs. By day 4 after hatching of the first chick, brood size was adjusted to either two or three chicks so that all T and C nests contained one senior chick of an unmanipulated egg (egg 1) and one or two junior chicks of injected eggs (eggs 2–6), while the father was invariably removed on that day (see above for rationale). We measured hatching time, body mass, body condition, and survival until fledging of the chicks and change in body mass, feeding rate, immunity, and features of subsequent clutches of mothers.

Animals and Housing Conditions

Adult zebra finches from a stock known to produce fertile eggs were housed in the facilities of the Zoological Lab-

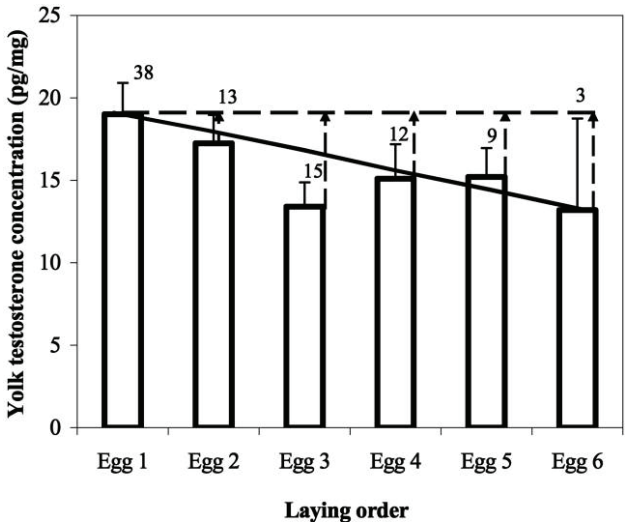

Figure 1: Within-clutch variation of yolk testosterone (T) level (mean + SE) detected in 90 freshly laid eggs of known laying order (*solid line*; sample sizes are reported over the bars) and experimental T increase induced in eggs 2–6 of T clutches after yolk injection (*dashed line*).

oratory of the Biological Centre of the University of Groningen, Netherlands, during March 2008. The room had a temperature of ~25°C, a relative humidity of ~40%–60%, and a 14L : 10D photoperiod (lights on at 9:00 a.m.). Pairs were housed in standard wooden cages (150 cm × 40 cm × 30 cm) with a metal grid front, two perches, sand on the floor, a nest box, and hay for nesting and were provided ad lib. with a mixed seed diet (Teurlings), water, cuttlebone, and grit. Food and water were refreshed every second day. The diet was supplemented with egg food and lettuce three times a week. Diet conditions were kept unchanged throughout each stage of the experiment.

Determination of Yolk T Concentrations over the Laying Sequence

Yolk T injections were based on levels of yolk T determined in 90 eggs of laying position 1–6 from 38 females of the same zebra finch population employed for the experiment. Yolk T was measured by radioimmunoassay following established protocols of extraction on celite columns (Wingfield and Farner 1975; Schwabl 1993), with slight modifications. Eggs were kept frozen at −20°C from the day of collection until extraction. For extraction, the yolk samples were thawed, weighed, and homogenized with 500 μL distilled water and a few glass balls. In order to assess and correct for the extraction efficiency of each individual sample, 50 μL tritiated T with known radioactivity (~2400–5400 dpm) was added to a weighed aliquot of ~150 mg homogenate (average recovery rate, 46.3%). The samples were extracted three times with 3 mL diethyl-ether/petroleum-ether (7 : 3 v/v), snap-frozen, decanted, and dried under a stream of nitrogen. Samples were redissolved in 1 mL of 70% methanol, frozen overnight at −20°C, and centrifuged and decanted to precipitate neutral lipids. The supernatant was dried under a stream of nitrogen and reconstituted in 1 mL of 2% ethylacetate in isooctane and transferred to 5-mL glass columns packed with 1.5 mL celite : propylene glycol : ethylene glycol (6 : 1.5 : 1.5 w/v/v) on top of a 0.5-mL water trap of celite : water (3 : 1 w/v). Steroids were eluted with 4 mL of pure isooctane (discarded), 4.0 mL of 2% ethylacetate in isooctane (discarded), 4.5 mL of 10% ethylacetate in isooctane (discarded), and 4.5 mL of 20% ethylacetate in isooctane (eluate containing T). The eluates were dried and redissolved in 200 μL Tris buffer. Testosterone levels were measured in duplicates of 50 μL of sample, using a DSL-4000 Coated Tube RIA Kit (Diagnostic Systems Laboratories), with a sensitivity of 0.08 ng/mL. Intra-assay variation was 7.1%, and interassay variation was 6.8% (based on 43 samples measured in two assays).

Yolk T Manipulation

Nests were checked once daily at 1 p.m. for eggs. All eggs in a clutch were weighed and marked at laying. Egg 1 was left untreated at the nest until hatching in all clutches. At the laying of egg 2, nests were randomly assigned to either a T or C treatment. The T treatment consisted of injecting the yolk of freshly laid eggs 2–6 with 5 μL of sterile sesame oil containing an amount of T that differed according to the laying order of the egg. The concentration of the solutions employed for eggs 2–6 was as follows: egg 2, 97.4 pg/μL; egg 3, 323.6 pg/μL; egg 4, 224.4 pg/μL; egg 5, 235.8 pg/μL; egg 6, 333.2 pg/μL. These dosages were chosen according to the results of the T assays (see above) to result in average yolk T concentration in eggs 2–6 of 19.01 pg/mg, which is the average concentration we detected at laying for egg 1 (fig. 1). The C treatment consisted of injecting eggs 2–6 with 5 μL of sterile sesame oil only.

Eggs 2–6 were injected with a 10-μL Hamilton syringe, according to the protocol by von Engelhardt et al. (2006). The procedure was, briefly, that eggs were illuminated from beneath and injected in the middle of the yolk at an angle of about 45° upward. Needle and eggshell were wiped once with 100% ethanol, and the eggshell was patched after injection with a tiny drop of paraffin. Eggs were put back in their nests immediately after the injection.

General Experimental Procedures and Composition of Broods

Females were weighed at day 4 after hatching of chick 1, when we removed the fathers from their cages. Thus, only the mothers were allowed to rear either T or C broods from day 4 to day 24 after hatching, the latter being the day when we put the fathers back in their cages. Date of laying of first egg and maternal body mass at day 4 did not differ between T and C nests (*t*-tests, $P > .22$ in all cases).

Just before the estimated hatching time (i.e., 13 days after laying of egg 1), eggs 2–6 of each clutch were moved until hatching to an incubator at 37°C and 50%–70% relative humidity and temporarily replaced by unfertile zebra finch eggs, collected from females kept in single-sex groups. Conversely, egg 1 was left in the nest and incubated by parents in both T and C groups. All eggs were inspected three times per day (every 7 h) to estimate hatching time. We obtained hatchlings from 19 T and from 18 C nests. Hatchlings were weighed and individually marked before putting them back in their original nests or, in a minority of cases, fostered in other nests. The latter was due to the fact that the hatching rate of unmanipulated (i.e., egg 1) and, especially, T and C eggs was lower than expected (i.e., egg 1, 83.8%; T eggs, 35.82%; C eggs, 35.48%; $N = 37$,

$N = 67$, and $N = 62$, respectively). As a consequence, in some clutches, no junior hatchlings followed the senior one, while in some others, the first egg did not hatch or more than two chicks hatched from eggs 2–6. In order to increase our sample size while adhering to our original design, we cross-fostered, whenever possible, supplementary T or C hatchlings in nests containing only the hatchling from egg 1, unless the age difference between the senior and the junior chicks was greater than 4 days. This schedule was adopted in order to maintain the hatching asynchrony of each brood within the natural range of variation for this species (Rutkowska and Cichoń 2005; Mainwaring et al. 2010; average hatching asynchrony in our sample, 46.00 h ± 8.13 SE). In this way, we could obtain 17 T broods, six of which included chicks of mixed origin, and 15 C broods, three of which included chicks of mixed origin. The T and C broods did not differ in the proportion of mixed broods (binomial test, $P > .50$) or in original clutch size (T, 4.71 ± 0.27 SE; C, 4.53 ± 0.13 SE), hatching date, or brood size at day 4 (T, 2.41 ± 0.12 SE; C, 2.33 ± 0.16 SE; *t*-tests, $P > .39$ in all cases).

Measurements of Chicks and Mothers

Measures on chicks and mothers were taken at standard days with respect to the day of hatching of the senior chick (day 0), that is, the chick from egg 1. Body mass of the chicks was measured at hatching, at day 12, and at day 24 after hatching of the senior chick; tarsus length was measured at day 12. Chicks were measured in the morning immediately after the lights were turned on, before they could be fed. Sex of surviving nestlings was determined by their adult plumage around day 35.

In addition, for the nests with at least the first chick and at least one junior chick surviving until day 12, we assessed maternal feeding rate at day 12 and subjected the mother to a standard in vivo test of her T cell–mediated immune response at day 15. Maternal feeding rate at day 12 (number of feeding visits to the nest and number of meals delivered per visit to the chicks) was determined from 2 h of videorecording using digital minicameras at the nest entrance. Recording sessions always started in the morning around 9.00 a.m., following the morphometrical measuring session at day 12. Day 12 was chosen because it represents the time when body mass and skeletal growth of the chicks are almost completed, hence, the end of the period in which mothers experienced the peak load in maternal care. Recordings were analyzed with VLC Media Player 0.8.4a. All measures were performed blindly with respect to the hormone treatment of the nest.

To assess maternal immune response at day 15, we injected 40 μg phytohemagglutinin dissolved in 0.04 mL of phosphate-buffered saline (PHA test; Saino et al. 1997) into the right wing web and measured to the nearest 0.01 mm the swelling due to infiltration of T-lymphocytes 24 h later by means of a spessimeter (Mitutoyo, 2046 F-60). Maternal PHA response was calculated as the change in thickness of the injected wing web over the 24-h period following injection, a larger swelling being taken as a larger, more competent immune response.

Subsequent Breeding of Mothers

Potential costs for future reproduction were assessed by allowing pairs to lay a new clutch following the reintroduction of original partners to their cages. The data on the breeding bout performed by the mothers after the reintroduction of their partner (latency in the onset of laying after partners' reintroduction, egg mass, clutch size, and clutch mass) were collected according to the same protocol adopted for the first breeding bout (see above).

Statistical Analyses

We used general linear mixed models (GLMM), with dependent variables being the measurements on hatching time, offspring, mothers, and subsequent breeding investment by mothers. In all models, the effect of the experimental treatment was tested by a fixed factor called nest treatment (T or C). Covariates were included to account for effects that could not be controlled experimentally, and interaction effects between covariates and nest treatment were entered only where there was a clear expectation. Random intercept and random slope effects were entered as specified below to account for nonindependence of observations from the same nest: for brevity, here we are only reporting statistics of random intercept effects for the main models; estimates of random slope effects were always very close to or equal to zero, thus never attaining significance.

Hatching time was tested only for eggs 2–6, including a random intercept for nest of origin and a random slope for the effect of egg position, depending on nest of origin. Egg position and its interaction with nest treatment were included to account for increasing dosage of T over the laying sequence in T nests, as well as for intrinsic differences in egg quality. Hatching mass was tested in the same model, with egg mass entered both as additional covariate and random slope effect to account for its strong positive effect on the dependent variable.

In all models of young, nest of rearing and nest of origin were included as crossed random intercept effects. In the analysis of chick body condition (i.e., body mass in relation to skeletal size) and chick survival at day 24, we entered a second fixed factor called egg treatment (injected or uninjected) to test for the between-groups effects of nest

treatment on senior (from uninjected first eggs) and junior (from injected eggs 2–6) chicks separately. Differential effects on senior and junior chicks in T and C nests were tested by the interaction term between nest and egg treatment. In the model on body condition, tarsus size was included as a covariate to account for variation in skeletal size across chicks, and its effect was allowed to vary randomly within both nest of origin and nest of rearing via random slopes. Chick survival at day 24 was coded as a two-state response variable (1 = surviving; 0 = not surviving) and tested in a logistic regression mixed model, assuming a binomial error distribution and a logit link function. In both analyses, initial brood size set by the experimenters by day 4 (two or three chicks) was subsequently included as a covariate to confirm previous results.

Models of maternal feeding rate at day 12 included nest treatment, brood size at day 12 (covariate), and their interaction, to account for differences in feeding effort imposed by variation in nest treatment and/or the number of chicks attended at the nest during recordings. All other models of maternal phenotype at day 12 included initial brood size and female mass at day 4 (i.e., initial female mass at removal of the male) as covariates, to control for differences in food demand by the offspring over days 4–12 and female quality at the beginning of the rearing period, which was unrelated to nest treatment. Models of subsequent breeding investment by mothers always included nest treatment, as well as initial brood size and the values of the variable under scrutiny recorded during the first breeding bout as covariates, because they were both likely to affect the dependent variables.

Offspring sex (male or female, fixed factor) was subsequently included in the analysis only on chick body condition at day 24, when sex of all but three chicks was known. Since dead embryos and hatchlings were not sexed, the data set was incomplete, and sex-specific effects on hatching time and offspring survival could not be analyzed.

All statistical analyses were run using the SAS (9.1) package. Parameter estimates were obtained by the restricted maximum likelihood method. Degrees of freedom were estimated by Satterthwaite approximation. Significance of fixed effects was assessed using *F*-tests or *t*-tests; significance of random effects was assessed using *z*-tests; post hoc tests were performed with Sidak correction. Residuals of the data met the conditions of normality and homogeneity of variances in all models (Kolmogorov-Smirnov and Levene tests, $P > .05$).

Results

Hatching Time, Offspring Mass, and Survival

The T eggs hatched significantly later than the C eggs, with a stronger effect for eggs early in the laying sequence (nest treatment × laying order analyzed for eggs 2–6, $F = 6.16$, df = 1, 30.3, $P = .019$; nest treatment, $F = 8.67$, df = 1, 36, $P = .006$; laying order, $F = 20.95$, df = 1, 30.3, $P < .001$; nest of origin, $z = 2.51$, $P = .006$; $N = 24$ T and 22 C eggs, untreated first eggs excluded; fig. 2). There was no effect of nest treatment or other predictors on hatching mass ($P > .410$ in all cases), taking into account a positive effect of egg mass ($F = 151.74$, df = 1, 41, $P < .001$, $b = 0.79 \pm 0.06$ SE; nest of origin, $z = .00$, $P = 1.00$).

At day 24, before fathers were reintroduced to the cages, junior, but not senior, offspring in T nests had a poorer body condition (low body mass in relation to their structural size) than did C chicks, as shown by a significant effect of the interaction of nest treatment and egg treatment on body mass in a model that included the last measured tarsus length (at day 12) as a covariate, to take structural size into account (table 1; fig. 3). Exclusion from the analysis of nests that included cross-fostered junior chicks (see "Methods") left these results unchanged (i.e., nest treatment and nest treatment × egg treatment interaction, both $P < .017$). Post hoc comparisons revealed that junior offspring hatched from eggs 2–6 had significantly lower size-corrected body mass in T compared to C nests ($P = .036$), whereas no difference was detected between the senior chicks (from egg 1) from the two groups ($P = .853$). All these results were confirmed when entering in the model either chick sex alone ($F = .78$, df = 1, 32.7, $P = .384$) or the chick sex × nest treatment × egg treatment interaction ($F = 13.30$, df =

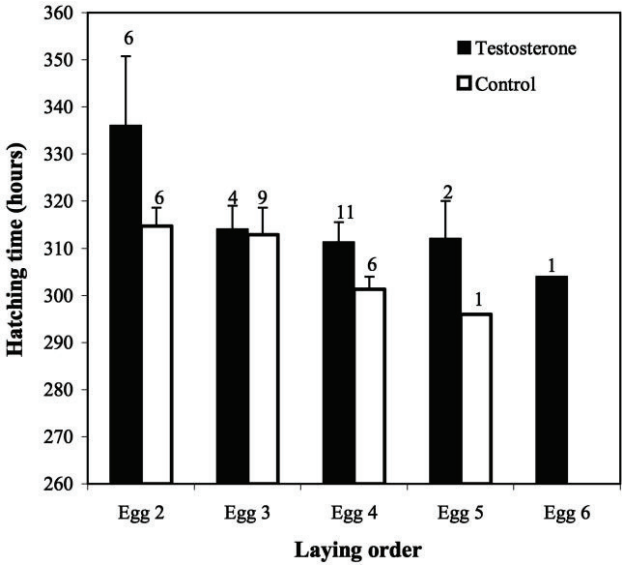

Figure 2: Hatching time (hours since laying, mean + SE) of 24 T (testosterone) and 22 C (control) eggs with respect to laying order. Sample sizes within laying order are also reported.

Table 1: GLMM analysis of body mass at day 24 in 31 T and 30 C offspring from 32 broods

	z	*F*	df	*P*	*b*	SE
Nest of rearing	.47			.319		
Nest of origin	1.00			.309		
Nest treatment		4.72	1, 23.8	.040		
Egg treatment		.06	1, 30.5	.814		
Nest treatment × egg treatment		6.93	1, 29.4	.013		
Tarsus length at day 12		117.5	1, 38.4	<.001	.54	.05

Note: The 31 T (testosterone) offspring were 15 seniors and 16 juniors; the 30 C (control) offspring were 12 seniors and 18 juniors.

1, 27.9, $P = .001$), the latter being mainly explained by a stronger detrimental effect of T treatment on junior females compared to junior males ($P < .001$; junior T males, day 24 body mass, 12.22 g ± 0.29 SE; junior C males, 12.76 g ± 0.29 SE; junior T females, 11.59 g ± 0.27 SE; junior C females, 13.16 g ± 0.30 SE).

Initial brood size did not affect body condition at day 24 ($F = 1.70$, df = 1, 23.7, $P = .205$). Tarsus size at day 12 ($P > .944$) or body mass at day 24 ($P > .103$) did not differ significantly between nest treatments when tested separately.

Survival of junior offspring in T broods was slightly lower than in C broods, whereas the reverse was the case for seniors, but the effect of the interaction between nest treatment and egg treatment did not reach statistical significance ($F = 1.59$, df = 1, 35, $P = .216$; nest of origin, $z = .00$, $P = 1.00$; nest of rearing, $z = 1.96$, $P = .025$; fig. 4). Inclusion in the model of initial brood size ($P > .748$) left these results unchanged.

Maternal Feeding Effort

Maternal feeding effort was analyzed at day 12, around the time of peak offspring growth and maternal workload, and for those broods that still had at least two or three chicks in the nest. The T mothers fed broods of two chicks more than did C mothers in such broods, while the opposite was true for broods of three chicks (table 2; number of feeding visits, see fig. 5; number of meals delivered, two-chick T nests, 96.86 meals ± 12.88 SE; two-chick C nests, 72.80 meals ± 28.38 SE; three-chick T nests, 84.67 meals ± 20.93 SE; three-chick C nests, 173.83 meals ± 31.15 SE).

Maternal Phenotype at the Peak of Parental Care

Between day 4, when fathers were removed, and day 12, mothers lost, on average, 1.01 g ± 0.19 SE, that is, ~7% of their body mass (paired-samples *t*-test, $t = 5.26$, df = 30, $P < .001$). There was no effect of nest treatment on maternal body mass at day 12 ($F = 0.24$, df = 1, 27, $P = .631$, taking female mass at day 4 ($F = 30.49$, df = 1, 27, $P < .001$) into account. Maternal body mass at day 12 also was not affected by initial brood size ($F = .46$, df = 1, 27, $P = .505$). Maternal immune responsiveness (PHA score) at day 15 was not affected by nest treatment ($F = .19$, df = 1, 28, $P = .666$) or female mass at day 4 ($F = .19$, df = 1, 28, $P = .665$) and did not differ between females rearing small or large broods ($F = .25$, df = 1, 28, $P = .623$).

Maternal Reproductive Output at the Subsequent Breeding Event

There were no significant effects of nest treatment on laying latency and mean egg mass of the next breeding event while correcting for the values recorded for these variables during the first breeding bout (table 3). However, there were a nonsignificant trend and a borderline significant effect, respectively, for females that had received T broods to lay smaller and lighter second clutches (table 3; clutch size, T nests, 4.38 eggs ± 0.31 SE; C nests, 5.17 eggs ± 0.33 SE; clutch mass, T nests, 4.96 g ± 0.36 SE; C nests, 6.02 g ± 0.38 SE).

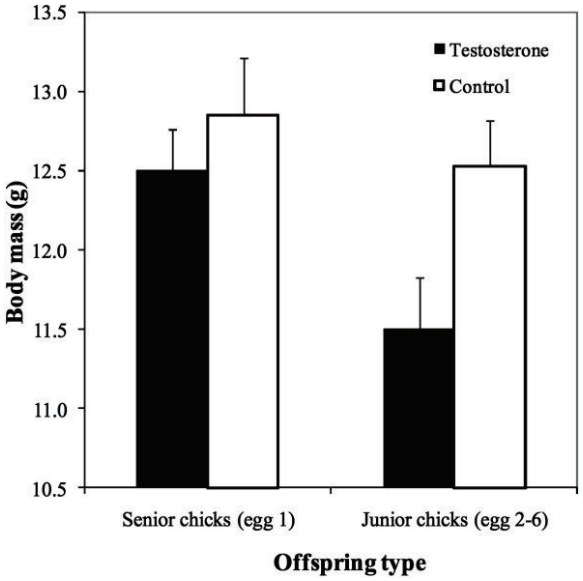

Figure 3: Day 24 body mass (g, mean + SE) of 31 T (testosterone) offspring (15 seniors and 16 juniors) and 30 C (control) offspring (12 seniors and 18 juniors) from 32 broods.

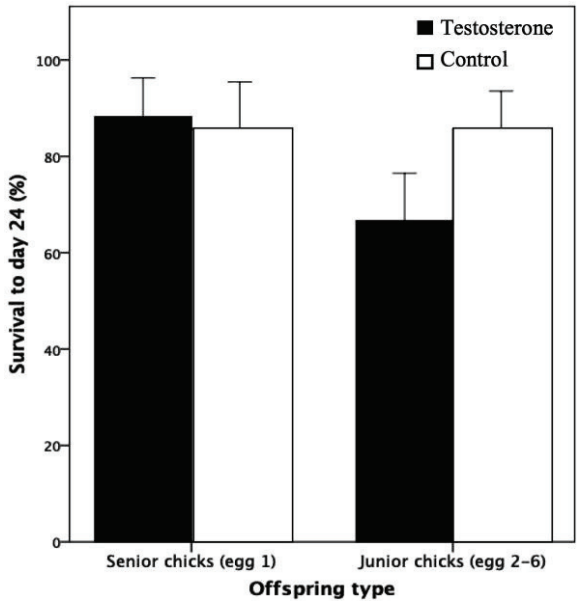

Figure 4: Within-nest survival to day 24 (%; mean + SE) in 17 T (testosterone) nests (10 normal and 7 large broods) and 15 C (control) nests (8 normal and 7 large broods).

Discussion

Parents should benefit from adjusting parental investment to offspring reproductive value. Individual offspring, on the other hand, benefit from obtaining more resources than is optimal from the parental perspective, which results in an evolutionary conflict between parents and offspring over parental allocation. The transfer of maternal hormones to offspring in several vertebrate taxa is thought to be an adaptive mechanism by which parents can optimize offspring development, but the question whether this maximizes maternal or offspring fitness, or both, has been largely neglected. Moreover, although it is generally assumed that differential allocation of maternal hormones among competing siblings provides the mother with a tool to adjust the sibling hierarchy, experimental evidence for this is scarce. In this study, we tested experimentally for the first time the hypothesis that mothers favor senior offspring in a brood over juniors by providing the first more exposure to maternal androgens than the latter. We tested this using the zebra finch, in which the eggs of the same clutch show a decrease in androgen concentration over the laying order, potentially benefiting first-hatched chicks over later-hatched siblings and therefore inducing a stronger conflict between mothers and junior chicks. To this end, we elevated yolk T levels of all eggs to the level of the first egg and analyzed the effects on offspring and mothers. Contrary to the hypothesis in the literature, the natural decrease of T over the laying sequence seems beneficial and not detrimental for junior chicks and perhaps also for their mothers, since junior chicks were negatively affected by elevation of yolk T levels, and a similar tendency was found for mothers, while senior chicks were unaffected.

The experimental treatment resulted in lower body condition at fledging of junior but not senior offspring in T compared to C broods, and there was weak evidence also for reduced survival of such chicks. Body condition is repeatedly found to correlate with winter survival or recruitment in wild birds (e.g., Alatalo and Lundberg 1986; Pettifor et al. 2001; Schmoll et al. 2003), suggesting that the effect of our treatment has a negative effect on the direct fitness of junior offspring. These negative effects may come about via two pathways. Prenatal exposure to experimentally elevated yolk T concentrations may increase energy demand by elevating metabolic rate (Tobler et al. 2007) to the extent that this cannot be fully compensated for by maternal workload. Accordingly, we found that maternal feeding effort at the peak of chick demand was reduced in large T broods. Alternatively, since the elevation of yolk T levels delays the hatching time in this species (this study; von Engelhardt et al. 2006), our treatment might have reduced the competitive ability of junior T chicks against seniors and thereby impaired their final condition (Cotton et al. 1999). In this case, reduced maternal workload in large T broods might have been determined by low quality of junior offspring, inducing the mother to spend less effort on these chicks. However, this is not in line with the slightly reduced performance of T mothers during the subsequent breeding bout.

The phenotype of senior offspring was not affected by

Table 2: GLM analysis of maternal feeding rate at day 12 in 13 T and 11 C nests

	F	df	*P*
Feeding visits:			
Nest treatment	9.68	1, 20	.006
Brood size at day 12	1.10	1, 20	.307
Nest treatment × brood size at day 12	11.68	1, 20	.003
Meals delivered:			
Nest treatment	4.35	1, 20	.050
Brood size at day 12	3.57	1, 20	.073
Nest treatment × brood size at day 12	5.80	1, 20	.026

Note: Maternal feeding rate = number of feeding visits and number of meals delivered at the nest within 2 h; GLM = general linear model; T = testosterone; C = control.

Figure 5: Maternal feeding rate (number of feeding visits within 2 h; mean + SE) at day 12 in 13 T (testosterone) nests (7 normal and 6 large broods) and 11 C (control) nests (5 normal and 6 large broods).

being reared in a T or a C nest. Hence, it is unlikely that, in zebra finches, decreasing T allocation over the laying order evolved to promote a direct benefit for senior offspring by favoring brood reduction or reinforcing competitive asymmetries determined by laying order. The unmanipulated senior chicks from T broods experienced an indirect fitness cost (sensu Hamilton 1964) instead, as the impaired quality of junior siblings was not compensated by adequate comparable enhancement of their own individual condition.

Although we showed that care of experimental broods was costly to the mothers in terms of substantial body mass loss during days 4–12, there was no evidence for direct costs of nest treatment on maternal phenotype during the nestling period. There was weak, marginally nonsignificant support for a possible delayed cost for mothers, since T mothers tended to lay smaller and lighter clutches during their subsequent breeding bout, thus suffering from potential fitness losses during future reproductive events because of lower fecundity compared to C mothers (Stearns 1992). If rearing T chicks was indeed costly, it is unclear how the effect was mediated, since overall, mothers seemed to invest less when rearing T chicks, as indicated by lower chick mass and reduced feeding rates, at least in large broods. To our knowledge, potential costs for mothers of rearing offspring exposed to experimentally altered levels of maternal hormones have not been assessed in other studies but should receive more attention, since this can be an important fitness component influencing the evolution of maternal effects mediated by hormones. The weak evidence emerging from our study despite ad lib. food availability suggests that under natural conditions, such effects may be exacerbated, potentially resulting in clear costs for mothers.

In our study, fathers were removed at the start of the experiment to avoid the effects of nest treatment being masked by having two parents attending relatively small broods and, furthermore, because compensatory adjustment of parental investment due to sexual conflict between the attending parents (Royle et al. 2002; Müller et al. 2007) might have confounded the results and made the interpretation of the study more difficult (see also "Introduction"). Although some observed effects, such as the delay in hatching time, do not depend on male removal, the particular experimental design should be taken into account when translating our results to the natural situation. With respect to the conflict existing between partners about the cost of reproduction (Trivers 1972), it would be very important to directly test the effect of resource allocation patterns adopted by the mothers on the behavior and fitness of fathers as well. Variation in yolk hormone allocation has even been considered as an evolutionary strategy adopted by avian mothers that increases paternal investment (Moreno-Rueda 2007; Müller et al. 2007), which is similar to the idea that paternal genomic imprinting in mammals and plants results in increased hormonal signaling by offspring to mothers and thereby increased maternal investment (Haig 1996). However, there currently is no experimental evidence for such an effect in birds (Ruuskanen et al. 2009).

Table 3: GLM analysis of the next breeding event in 17 T and 15 C nests

	F	df	*P*	*b*	*SE*
Latency to lay:					
Latency to lay clutch 1	.07	1, 28	.794	−.04	.15
Nest treatment	.26	1, 28	.613		
Initial brood size	.54	1, 28	.470	1.17	1.59
Mean egg mass:					
Mean egg mass brood 1	92.50	1, 28	<.001	.95	.10
Nest treatment	.13	1, 28	.725		
Initial brood size	.95	1, 28	.344	−.02	.03
Clutch size:					
Clutch size brood 1	.53	1, 28	.474	.20	.27
Nest treatment	3.00	1, 28	.095		
Initial brood size	2.09	1, 28	.160	−.67	.46
Clutch mass:					
Clutch mass brood 1	.83	1, 28	.370	.21	.23
Nest treatment	4.14	1, 28	.051		
Initial brood size	.55	1, 28	.466	−.41	.56

Note: Next breeding event = latency of laying, egg mass, clutch size, and clutch mass; GLM = general linear model; T = testosterone; C = control.

With respect to our initial predictions, we therefore conclude that the decreasing pattern of T allocation adopted within clutches by zebra finch mothers is directly beneficial for junior offspring and indirectly benefits senior offspring and mothers. Our results add new perspectives concerning the adaptive within-clutch modulation of prenatal allocation of resources among competing offspring and adaptive maternal effects in general. The hypothesis that increasing and decreasing allocation patterns reflect a brood survival and a brood reduction strategy, respectively, may be too general and may depend on the species and its ecological context (e.g., Sockman and Schwabl 2000 vs. Eising et al. 2001). The generality of the hypothesis is based on the assumption that effects of yolk T would be similar for all bird species, but this clearly is not the case and adds an additional layer of complexity to the interpretation of hormone-mediated maternal effects. For example, as we have demonstrated here, yolk T delays hatching time in the altricial zebra finch, confirming results of an earlier study (von Engelhardt et al. 2006), while it speeds up hatching time in the semiprecocial black-headed gull (Eising et al 2001, confirmed by Eising and Groothuis 2003). Such species-specific effect of elevated yolk T may be caused by an interaction with other yolk components that needs further study (Groothuis et al. 2005; Gil 2008), and/or derive from species-specific adaptation in the response of the offspring to androgen allocation by the mothers as the result of parent-offspring conflict, a topic that only recently emerged in the literature (Müller et al. 2007; Kilner and Hinde 2008; Tobler and Smith 2010). In any case, it warrants much more caution when reviewing the literature of maternally derived yolk androgens than is currently given.

Finally, we strongly encourage future research in this field to measure both direct and indirect consequences of parental effects on all family members and to be more cautious when drawing conclusions from published studies if consequences have not been measured exhaustively among family members with potentially conflicting interests.

Acknowledgments

We thank J. van Alphen for help with the experiment and three anonymous reviewers for helpful comments. This study was performed according to Dutch laws on animal research (DEC authorization number: 5237A). G.B. was funded by a postdoctoral grant from the Ministero Italiano dell'Università e Ricerca and by Marie Curie Intra-European Fellowship PIEF-GA-2009-252120 and N.V. by Marie Curie Intra-European Fellowship MEIF CT-2006-039362.

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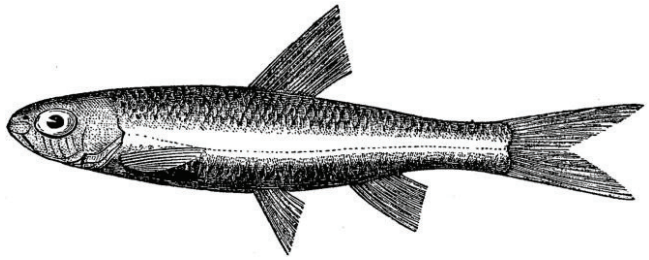

"On submitting this cyprinoid figured above to Professor Cope of Philadelphia, he pronounced it undescribed and has since described it as *Hybognathus osmerinus*. . . . Of its habits, as yet, we have determined nothing." From "Further Notes on New Jersey Fishes" by Charles C. Abbott (*American Naturalist*, 1871, 4:717–720).