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Influence of mate preference and laying order on maternal allocation in a monogamous parrot species with extreme hatching asynchrony

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Abstract

It is well established that in many avian species, prenatal maternal resource allocation varies both between and within clutches and may affect offspring fitness. Differential allocation of maternal resources, in terms of egg weight and yolk composition, may therefore allow the female to adjust brood reduction and to fine-tune reproductive investment in accordance with the expected fitness returns. The adaptive value of such maternal resource allocation is thought to be context-dependent as well as species-specific. We investigated the effects of female preference for her mate on the allocation of prenatal maternal resources in the budgerigar, *Melopsittacus undulatus*, a monogamous species of parrot that shows an extreme hatching asynchrony. We assessed mate preferences in a two-way preference test and allowed females two breeding rounds: one with the preferred and one with the non-preferred partner. We found no effect of preference on either latency to lay or clutch size, but females mated with the preferred partner laid eggs that contained significantly more yolk. Their eggs also contained significantly more androstenedione but not testosterone. Our results suggest that in this species, female preference may influence maternal resource allocation, and that the functional roles of each androgen in the yolk should be considered separately. In addition, we found a significant effect of laying order on egg and yolk weight as well as on yolk testosterone and androstenedione levels. These measures, however, did not change linearly with the laying order and render it unlikely that female budgerigars compensate for the extreme hatching asynchrony by adjusting within-clutch allocation of prenatal maternal resources.

Keywords: Maternal effects, Differential allocation, Male attractiveness, Yolk hormones, Hatching asynchrony, Androstenedione, Testosterone, Reproductive investment, *Melopsittacus undulatus*
Introduction

Early maternal allocation of resources provides a non-genetic mechanism for the female to modify the phenotype of the offspring in accordance with the environmental conditions that she experiences and that her offspring are likely to face after hatching (Mousseau and Fox, 1998). In oviparous animals, such as birds, the egg provides a sealed system in which the embryo develops and once the egg is laid, investment in the offspring through maternal resources is restricted. Birds are therefore excellent subjects for studying the causes and consequences of differential maternal allocation during the prenatal phase.

In birds, maternal resources that determine egg quality include egg weight and various yolk compounds (e.g. antibodies, antioxidants, androgens; Blount et al., 2002; Groothuis et al., 2005a; Krist, 2011; Müller et al., 2004; von Engelhardt and Groothuis, 2011). These maternal resources may vary both within and between clutches. Within clutches, species-specific deposition patterns of maternal resources over the laying order have been documented for several species (e.g. androgen deposition and egg weight; Groothuis et al., 2005a; Muller and Groothuis, 2013; Slagsvold et al., 1984). This has typically been interpreted in the context of hatching asynchrony, which is caused by the fact that females may lay more than one egg, but eventually start incubating before the clutch is completed. The resulting hatching spread and associated size hierarchy in the nest negatively affect the survival of chicks from later-laid eggs as they experience a competitive disadvantage compared to their older siblings (Massemin et al., 2002). It has been proposed that differential allocation patterns of maternal resources within a clutch enable the female to adjust brood reduction, thereby maximizing her own fitness (Schwabl, 1996; Wagner and Williams, 2007). An increase in maternal allocation with the laying order may counteract the competitive disadvantage of the chicks from later-laid eggs, because chicks that hatch from eggs with heavier yolks or higher levels of the maternal androgens androstenedione (A4) and/or testosterone (T) may hatch earlier, beg more vigorously, grow faster and have higher survival rates (e.g. Eising et al., 2001; Groothuis et al., 2005a; Krist, 2011; Schwabl, 1996; von Engelhardt and Groothuis, 2011; Wagner and Williams, 2007). A decrease in resources on the contrary may further enhance brood reduction (Groothuis et al., 2005a). However, the situation may be more complex, as in some species certain maternal resources such as yolk androgens have mainly negative effects on offspring fitness (Groothuis et al., 2005a, b; von Engelhardt and Groothuis, 2011). Together with the fact that species vary considerably with respect to within-clutch allocation patterns, this suggests that the adaptive value of maternal
allocation is species-specific and may differ according to the type of resource. To increase our understanding of the phylogenetic and life-history factors that potentially underlie within-clutch allocation patterns, studies that measure different parameters of egg quality in a large variety of species of different taxa are necessary (Gil et al., 2007; Love et al., 2009).

Variation in maternal allocation between clutches has been attributed to a range of environmental determinants and factors intrinsic to the female (e.g. social density, food availability, condition, age; Christians, 2002, Hargitai et al., 2009; Remes, 2011; Safran et al., 2008, 2010; Vergauwen et al., 2012; reviewed in: von Engelhardt and Groothuis, 2011). Reproduction is costly for the female, and there is thus a trade-off between investment in the current reproductive attempt and future reproduction (Williams, 1966). Hence, females are thought to adjust their reproductive effort in accordance to the expected fitness return. One important factor that may influence the reproductive decisions of the female is mate quality (Burley, 1988). Females may experience higher fitness returns when investing more heavily in reproduction when mated with a more attractive male (differential allocation hypothesis; Burley, 1988; Sheldon, 2000), because attractive males may provide more direct benefits such as parental care or territory quality and indirect benefits in terms of offspring genetic quality (Andersson, 1994). The effects of male attractiveness on maternal allocation have been examined in a variety of species, but the resulting data are inconclusive (reviewed in: Cucco et al., 2011; Horvathova et al., 2012; Kingma et al., 2009). Interestingly, in most studies, male attractiveness was determined based on previous work and it was not specifically tested whether females indeed preferred the males that were considered to be more attractive (e.g. Alonso-Alvarez et al., 2012; Garcia-Fernandez et al., 2013; Gil et al., 1999, 2004; Grana et al., 2012; Kingma et al., 2009; Safran et al., 2008; but see: Bluhm and Gowaty, 2004; Cucco et al., 2011; von Engelhardt et al., 2004). In order to estimate male attractiveness as perceived by the female, it is necessary to allow females to actively choose between potential partners.

The budgerigar, *Melopsittacus undulatus*, is a small monogamous species of parrot (Juniper, 1998). Female budgerigars of self-selected pairs show a shorter latency to lay and produce larger clutches (Massa et al., 1996), which indicates that mating preferences may be an important factor influencing maternal allocation. It has not yet been investigated whether females flexibly adjust maternal allocation of resources, such as yolk weight and androgen deposition, according to mate preference. Unlike many other bird species, parrots produce clutches with extreme hatching asynchrony (budgerigar broods hatch over a span of up to 16 days). Despite the apparent lower competitive abilities of younger chicks, brood reduction is uncommon (Brockway, 1964; Stamps et al., 1985). To the best of our knowledge, it has not
been reported in the literature whether or not female budgerigars compensate for the competitive disadvantage of younger chicks as caused by the extreme hatching asynchrony via differential prenatal maternal allocation to the eggs, as has been found for many species with less pronounced asynchrony.

In the current study, we assessed female preferences in a two-way choice test (von Engelhardt et al., 2004). Subsequently, the females were allowed two consecutive breeding rounds in a cross-over design. In one breeding round, they were paired with their preferred partner (i.e. the more attractive male as perceived by the female) and in the other with the non-preferred partner (i.e. the less attractive male as perceived by the female). Our aims were twofold. First, we tested whether females increase the allocation of resources when they are paired with the preferred partner by studying the effects of female preference on latency to lay, clutch size and the following six maternal resources that have been shown to influence egg quality: egg weight, yolk weight and the concentrations and the total amounts of the androgenic hormones T and A4 in the yolk. Finally, we asked whether maternal allocation varies over the laying order by investigating within-clutch variation in these six maternal resources.

Material and methods

Ethics statement

The budgerigars of this study were domesticated animals, which were used to human presence. Because budgerigars are social birds, we always allowed the birds at least vocal interactions with each other. We did not observe abnormal behavior performed by any of the birds during the experimental procedures or during the daily routine checks. Handling time was minimized and did not exceed 3 min per individual for any of the procedures. All experimental procedures were performed in agreement with the Belgian and Flemish laws.

Study species and housing

We randomly selected 39 unpaired female and 76 unpaired male budgerigars from our captive stock population. The birds had been obtained from local breeders as juveniles and had been maintained in our captive stock for and least one and up to two years. All females showed a green plumage and were adults of approximately one year old with no breeding experience. The males varied in plumage coloration and were approximately between one and two years
old. Before the experiment, the birds had been housed in two single-sex outdoor aviaries (8m wide x 2.5m deep x 2.3m high). One week before the start of the preference tests (see ‘Preference tests’), the birds were moved to indoor single-sex cages (males: 120cm wide x 40cm deep x 50cm high, females: 150cm wide x 40cm deep x 80cm high). The birds were housed in groups of up to 10 individuals and there was no visual or auditory contact between members of the opposite sex. Throughout the experiment, the birds were maintained on a light regime of 15:9 (L:D), unless stated otherwise. Food (commercial budgerigar seed mix, Nifra Van Camp bvba, Belgium), greens and water were provided ad libitum.

**Male stimulus sets**

The females were allowed to choose between two males of a stimulus set (see ‘Preference tests’). The males of a stimulus set were matched for body weight. We also matched males with higher and lower UV reflectance of the chest feathers, because female budgerigars may show mating preferences based on this trait (Griggio et al., 2010a; Zampiga et al., 2004). The color of the chest was measured with an USB4000 spectrophotometer (Ocean Optics, Duiven, The Netherlands), using an Ocean Optics DH-2000 BAL deuterium/halogen lamp. Before the measurement session, we took a dark current measurement on the chest of a randomly selected live bird and a white standard reference measurement (WS-1, Diffuse Reflectance Standard, Ocean Optics, Duiven, The Netherlands) for calibration purposes (Cuthill et al., 1999; Lahaye et al., 2014). Next, the chest of all males was measured three times by the same person (Lahaye et al., 2014). From the measurements, we calculated UV chroma as the proportion of total reflectance occurring between 320-400nm ($R_{320-400}/R_{320-700}$) (Montgomerie, 2006). For each male, we first calculated UV chroma separately for the three spectra that were measured, and the mean of these three values was used in the statistical analyses (Lahaye et al., 2014). Each stimulus set contained a male with a higher value for UV chroma of the chest (mean: 0.302 ± 0.0031) and a male with a lower value (0.255 ± 0.0027). The males of a stimulus set showed a mean difference of 0.048 ± 0.0017 (15.73 ± 0.49%) for UV chroma of the chest, which was statistically significant (paired t-test: $t_{26}$=-28.9, $P<0.0001$). A difference of this size is detectable by females of this species (Griggio et al., 2010a; Zampiga et al., 2004) and is therefore biologically relevant.

**Preference tests**

Female preferences were assessed in a two-way choice test during six consecutive days (May 27 – June 1, 2010). Our preference test protocols have been described previously (Lahaye et
al., 2013). Briefly, ten preference tests were conducted simultaneously in one single room between 0800 and 1300 hours local time. The preference test apparatus consisted of two small cages (35cm wide x 35cm deep x 50 cm high) which each contained one male. The small cages were placed in front of a large central cage (120cm wide x 40cm deep x 50cm high; fig. 1) in which a female was housed. Each male had access to one perch. The female had access to three perches: one perch in the center of the cage (i.e. no-choice area, fig. 1) and one perch in front of each stimulus male (i.e. choice area, fig. 1). The males were visually separated and the female could only see the males when she was positioned in the choice area, preventing her from seeing the males simultaneously and from seeing the males while she was positioned in the no-choice area (Griggio et al., 2010a, fig. 1). All individual preference test apparatus were visually separated from each other. The experimental cages were illuminated by artificial full spectrum light (Philips True Light, 58W/5500) and natural light through windows. A similar apparatus has been used in previous studies investigating mating preferences in a variety of species, including budgerigars (e.g. Griggio et al., 2010a; Lahaye et al., 2013; Moravec et al., 2010; Rutstein et al., 2007).

The afternoon before each preference test, the birds were moved to the preference test apparatus. The birds were visually, but not acoustically, isolated, and they were allowed to adjust to the new environment overnight. The following morning, the artificial lights were turned on, the partitions that visually separated the female from both stimulus males were removed, and the birds were allowed a habituation period of 50 min (von Engelhardt et al., 2004). After 50 min, the position of the males was switched, and a video recording of 50 min was made using digital camcorders (DCR-SX30E, Sony, Tokyo, Japan). During preliminary testing of our apparatus, we noticed that in many cases the females initially showed little mobility and only interacted with one stimulus male without visiting the second. By switching the position of the males before the first recording, we could increase the probability that all females had interacted with both males at the start of the actual test phase. A second 50-min recording was made, after switching the position of the males again to control for side preferences of the female.

Preliminary testing of our apparatus also revealed that the females often stayed immobile for up to 30 min after the disturbance caused by switching the position of the males. Highly social species, such as the budgerigar, may cope less well with disturbance when they are housed individually, which was the case during our preference tests (Cohen and Wills, 1985). Therefore, we analyzed 15 min of each recording, excluding the first 30 min and last 5 min to avoid potential disturbance caused by manually switching the test cages, by entering
and leaving the testing facility, and by starting and stopping the cameras. We calculated a preference score by scoring the position of the female every 10 seconds (left choice area, right choice area or central), yielding a total of 90 score points per video recording. We calculated the total association score for a male by summing the score obtained for the males over the two video recordings of a preference test. This score was used in further data analysis. We considered a preference test to be successful if the female was responsive during the preference test (i.e. the female spent at least 60% of the choice period (≥108 score points) in the left or right choice area; Enstrom et al., 1997; McGlothlin et al., 2004) and if the female exhibited a preference (i.e. the preference score for both males differed significantly; one-tailed binomial test, \( P<0.05 \)). Females that failed to meet these criteria were re-tested with a different set of stimulus males. The male stimulus sets were used to test the preferences of up to three different females. Only females that met our criteria were included in the experiment. A total of 11 females showed a significant preference during their first preference test. We re-tested the preference of 21 females and in their second preference test, 16 females showed a significant preference, resulting in a total of 27 females that met our criteria. Females were tested with unrelated, unfamiliar males. All females present in the testing room at any time were unfamiliar with all males that were present in the same room.

Pairing

We assessed female preferences from the video recordings within two hours of the preference test. Subsequently, females that showed a significant preference were paired with either their preferred male or their non-preferred male by moving the male into the central cage with the female. The second male (i.e. the other male, that was part of the male stimulus set which was presented during the preference test, but that was not moved into the central cage with the female) remained visible in the small cage for one hour to increase the time the female was exposed to both the preferred and non-preferred male (von Engelhardt et al., 2004). After one hour, the paired birds were moved into a breeding cage in the breeding facilities (see ‘Breeding’). In our experimental design, we applied a statistically strong cross-over experimental design. A total of 15 females were first paired with the preferred male and subsequently with the non-preferred one, and 12 females the other way around. Therefore, the second male of the female’s preference test, was kept apart in the single-sex outdoor aviary, to be used in the second breeding round.
Breeding

Following the preference tests, the pairs were moved to the breeding facilities. All pairs were housed individually in breeding cages (60cm wide x 40cm deep x 50cm high) supplied with a nestbox (15cm wide x 15cm deep x 25cm high). Because the budgerigar is a highly social species which breeds colonially, breeding success may be low when individual pairs are deprived of auditory and visual contact with other breeding pairs (Brockway, 1964; Wyndham, 1981; our personal observation). Hence, all pairs were housed in separate breeding cages but in one single room, and we allowed the pairs auditory and visual contact to increase breeding success. After the introduction of the pairs, the nestboxes were inspected daily for the presence of eggs. Latency to lay was calculated as the number of days between the pairing of the birds and the appearance of the first egg. Eggs were collected within 24 hours after laying and replaced by plastic dummy eggs to avoid interruption of egg laying. All eggs were individually marked using a non-toxic pen, measured, weighed and frozen at -20°C until hormone analysis. Upon collection of the first clutch, the male was removed from the breeding cage. After five days, the second male from the female’s preference test (see ‘Pairing’) was introduced into the breeding cage and the female and the second male were allowed to produce a clutch (Gil et al., 2004). The males from the pairs that had failed to produce a clutch after one month were also removed from the breeding cages and, after five days, they were replaced with the second male from the female’s preference test. The collecting and handling of the eggs of the second clutch were done identically as for the first clutch. Upon completion of the second clutch, or eight weeks after the introduction of the second male, all pairs were separated and the birds were put back in our captive stock.

Hormone assay

Our protocols to measure the concentrations of T and A4 in the yolk have been described previously (Goerlich et al., 2009). Briefly, yolks were weighed to the nearest 0.001g, 1:1 diluted with dematerialized water and homogenized thoroughly. We then weighed ca. 200 mg of each yolk mixture for extraction. Prior to the extraction we added ca. 5000 cpm radioactive labeled T to each sample in order to account for losses due to the extraction procedure. Each sample was extracted three times with 2.0 mL diethyl ether/petroleum benzine 70:30 (DEE/PB, vol:vol). After vortexing (60 sec), centrifuging (5 min, 2000 rpm, 4°C) and snap freezing, the organic phase was decanted and the extract was dried under a stream of nitrogen. The extraction procedure was repeated twice with an additional 2.0 mL of DEE/PB, vortexed for 30 sec and 15 sec, respectively. Next, the extracts were dried under nitrogen. Hormone
extracts were rinsed in 2.0 mL of 70% methanol to precipitate any lipids. The samples were vortexed until the complete dried pellet was dissolved. Samples were frozen at -20°C overnight and then centrifuged (5 min, 2000 rpm, 4°C), decanted and dried. The pellet was re-dissolved in 600 µL phosphate buffered saline with gelatine (PBSG). Recoveries of the initially added labeled T were measured in a subsample of this solution. Average recovery was 77.6 ± 0.3% (mean ± SE). Concentrations of T and A4 in the yolk were determined using commercial Radioimmunoassay (RIA) kits (Spectria® T Coated-Tube RIA kit, Orion Diagnostica Espoo, Finland; detection limit: 0.03 ng/mL, antibody cross-reactivity: 100% T, 4.5% 5α-dihydrotestosterone, and 0.007% A4; Active® A4 Coated-Tube RIA DSL-3800 kit, Diagnostic Systems Laboratories, Beckman Coulter, The Netherlands; detection limit: 0.03 ng/mL, antibody cross-reactivity: 100% A4, 0.33% Androsterone, and 0.08% 5α-dihydrotestosterone). Samples were distributed over two RIAs for T as well as A4 such that all samples from one female were measured within the same assay and with both preference groups (i.e. females that were first paired with the preferred and subsequently with the non-preferred partner and vice versa) distributed equally. The assay kits were validated by ensuring parallelism of serial dilutions of four random yolk samples with the standard curve. For A4 there was no perfect parallelism, so a correction factor was calculated by dividing the slope of the standard curve by the average of the slopes of the four serial dilutions. RIA concentrations were corrected for initial yolk mass and calculated as pg/mg yolk. We included a selection of pool samples in both assays to estimate intra-assay variability (T-assays: n=24; A4-assays: n=15). The average intra-assay coefficients of variation (CV, (stddev(avrg)/avrg)×100) were 2.15% for T, and 2.51% for A4. Repeatability for the pool samples measured in both assays was very high for both T and A4 (r>0.95, P<0.0001 in both cases; Lessells and Boag, 1987).

Data Analysis

We analyzed all data using the statistical package SAS® 9.3 (SAS Institute, Cary, NC, 2008). Because we did not know whether the concentrations or the amounts of androgens in the yolk are biologically more relevant in our study species, we modeled (i) concentrations, and (ii) total amounts of both T and A4 in the yolk, obtained by multiplying concentrations of respectively T and A4 by yolk mass. Six eggs of six different clutches (one first, second, third and fifth egg and two fourth eggs) were not included in the analyses because they were broken in the nest or because the egg contained two yolks.

We performed Pearson’s correlations to investigate the relationships among the six
types maternal resources of a clutch that were measured in this study (mean egg and yolk weight, mean concentrations and total amounts of both T and A4 in the yolk). We used a mixed modelling approach to examine the effects of mate preference on maternal allocation. In each of the six models, we included preference (clutch produced with preferred or non-preferred male), clutch (1-2) and laying order (egg 1-8) as fixed factors and clutch size (3-8) as a covariate. Laying order was fitted as a categorical variable because the effects of laying order may not be linear (Kozlowski and Ricklefs, 2010; Salomons et al., 2006), which is of particular interest within the scope of our research aim. We also included two-way interactions between the fixed effects (see table 1 and 2). Our dataset contains two levels of nested random effects, as a series of eggs were collected in two clutches of the same female. We nested individual egg within clutch as a random coefficient for laying order and clutch within female as a random coefficient for preference (cf. random slopes model; Schielzeth and Forstmeier, 2009). We also included female condition (body weight) as a covariate in all models, but this factor was not significant in any of the models ($P > 0.3$ in all cases) and thus we excluded the variable from all models. To analyze the effects of preference on latency to lay and clutch size we used two similar mixed models with preference, clutch and the preference*clutch interaction term as fixed effects. Both models were corrected for the random coefficient of clutch, nested within individual female. Several covariance structures (i.e. compound symmetry, serial autocorrelation and unstructured) were tested in all the above models to select the best fitting regression based on Bayesian information criterion (BIC) values, which turned out to be the compound symmetry model in all cases. All response variables were normally distributed (Shapiro-Wilk, all $W \geq 0.90$), except latency to lay which had to be log-transformed to meet this model assumption ($W = 0.95$). We always started with the full model and subsequently excluded, step by step, all non-significant terms. At each step, the term that gave the smallest contribution (largest $P$-value) was excluded. Eta-squared effect sizes ($\eta^2$) were calculated as the ratio of variance explained by a predictor based on the final model, thus only including significant terms. Similarly, effect size of the covariate (clutch size) is estimated by the regression coefficient ($r$). Effect sizes for pair-wise comparisons are given as Cohen’s $d$, i.e. as the difference between two means divided by the standard deviation. Values are consistently reported as mean ± SE and a significance level of $P < 0.05$ was used throughout.
Results

Female preference, latency to lay and clutch size

A total of 27 females showed a significant preference for one of the two males ($P<0.025$ in all cases, fig. 2). The male with the higher value for UV chroma of the chest was preferred by 17 females, while 10 females associated more with the male with the lower value (one-tailed binomial test: $X^2=1.82, P=0.18$, fig. 2). A total of 16 females produced a clutch with both males. Four females produced only one clutch; two with the preferred and two with the non-preferred male. Seven females did not lay eggs with either male (fig. 2).

The first egg was laid between 9 and 43 days after pair formation (18.47 ± 1.46 days). Latency to lay was not significantly affected by preference ($F_{1, 17}=0.34, P=0.57$; preferred: 18.83 ± 1.97 days; non-preferred: 18.11 ± 2.21 days) or clutch ($F_{1, 17}=1.67, P=0.21$; first clutch: 19.05 ± 1.26 days; second clutch: 17.75 ± 2.94 days) and the preference*clutch interaction term was also not significant ($F_{1, 18}=0.23, P=0.63$).

Clutch size varied between 3 and 8 eggs (6.03 ± 0.19 eggs). For females with the preferred partner, the average clutch size for the first clutch was 6.17 ± 0.42 eggs ($n=12$; of which two females produced only one clutch) and for the second clutch 6.66 ± 0.21 eggs ($n=6$ females). For females with the non-preferred partner, the average clutch size for the first clutch was 5.45 ± 0.45 eggs ($n=8$ females, of which two females only produced one clutch) and for the second clutch 5.90 ± 0.32 eggs ($n=10$ females). There was no significant effect of preference on clutch size ($F_{1, 14}=0.62, P=0.44$). There was also no significant effect of clutch ($F_{1, 12}=0.12, P=0.74$) and the preference*clutch interaction term was also not significant ($F_{1, 14}=0.28, P=0.61$).

Egg weight and yolk weight

Mean egg weight and yolk weight of a clutch were positively correlated ($r=0.63, P<0.0001, n=36$ clutches). We found a significant overall effect of preference on yolk weight ($\eta^2=0.05$), but not on egg weight (table 1, fig. 3a and 3b). Yolk weight of the second clutch was significantly higher (table 1, fig. 3a and 3b; $\eta^2=0.02$). Clutch size significantly affected both egg weight and yolk weight (egg weight: $r=0.41$; yolk weight: $r=0.21$). Larger clutches contained heavier eggs with more yolk (table 1, fig. 4a). None of the interactions were significant for egg weight and for yolk weight, there was only a significant effect of the preference*clutch interaction term (table 1; $\eta^2=0.08$). Tukey adjusted post-hoc tests revealed that females that were paired with the preferred partner for the second clutch produced eggs
with the heaviest yolks (compared to the same females with the non-preferred partner for the first clutch: $t_{72}=-3.54$, $P_a=0.004$, $d=0.53$, 95% CI [0.09-0.96]; and compared to females with the preferred partner for the first clutch: $t_{72}=-2.92$, $P_a=0.024$, $d=0.87$, 95% CI [0.46-1.29] and the non-preferred partner for the second clutch: $t_{72}=2.66$, $P_a=0.047$, $d=0.71$, 95% CI [0.28-1.13]).

**Yolk androgens**

With regard to both concentrations and total amounts, we found that the yolk contained on average approximately five times more A4 than T, irrespective of treatment (fig. 3c-f). Mean concentrations of T and A4 in the yolks of a clutch were positively correlated ($r=0.80$, $P<0.0001$, n=36 clutches) and this was also the case for mean total amounts of T and A4 in the yolk ($r=0.85$, $P<0.0001$, n=36 clutches). Mean concentrations of T and A4 in the yolks of a clutch were not correlated with either mean egg weight (T: $r=0.27$, $P=0.11$; A4: $r=0.10$, $P=0.57$, n=36 clutches) or mean yolk weight of that clutch (T: $r=-0.044$, $P=0.80$; A4: $r=-0.25$, $P=0.15$, n=36 clutches). Both mean total amounts of T and A4 in the yolk were positively correlated with mean egg weight as well as mean yolk weight of a clutch ($r>0.45$, $P<0.01$ in both cases, n=36 clutches).

For concentrations and total amounts of T in the yolk, no significant effect of preference was found (table 2, fig. 3c and 3e). Eggs laid by females paired with the preferred partner contained significantly higher total amounts and tended to contain higher concentrations of A4 in the yolk (table 2, fig. 3d and 3f; total amounts: $\eta^2=0.02$; concentrations: $\eta^2=0.02$). The concentrations of T in the yolk, but not the total amounts, were significantly higher in the first clutch (table 2, fig. 3c and 3e; $\eta^2=0.03$). No significant effect of clutch was found for A4 (table 2, fig. 3d and 3f). Clutch size significantly affected maternal androgen deposition (table 2). The yolks of larger clutches contained significantly higher concentrations and total amounts of both T and A4 (fig. 4b and 4c; concentrations of T: $r=0.29$; total amounts of T: $r=0.33$; concentrations of A4: $r=0.36$; total amounts of A4: $r=0.43$). For concentrations and total amounts of A4 in the yolk, we found a significant preference*clutch size interaction term, which indicates that the effect of preference was stronger for larger clutches (table 2, fig. 4c). None of the other interaction terms were significant (table 2).
**Effects of laying order**

There was a significant effect of laying order for all six types of maternal resource that we measured in this study (tables 1 and 2). The second egg of a clutch contained a significantly heavier yolk than the fifth, sixth and seventh egg ($t_{96}>3.20$, $P<0.042$ and $d>0.46$, in all cases, fig. 5a) and the third egg contained a heavier yolk than the sixth and seventh egg ($t_{96}>3.40$, $P<0.02$ and $d>0.49$, in both cases, fig. 5a). For egg weight, only egg 2 was significantly heavier than egg 6 ($t_{96}=3.12$, $P=0.047$, $d=0.24$, 95% CI [-0.26-0.74], fig. 5a). For both T and A4, the values for concentrations as well as total amounts in the yolk were lower in the first egg compared to the second egg and then remained more or less stable or gradually declined from the second egg on (fig. 5b and 5c). The effects of laying order were not affected by preference, clutch or clutch size (tables 1 and 2).

**Discussion**

In this study, female budgerigars were allowed to choose between two males, and we subsequently investigated the effects of female preference for her mate on maternal allocation of resources to the eggs. Additionally, we tested whether females increase allocation of resources to the eggs with the laying order to compensate for the competitive disadvantage of younger chicks due to the extreme hatching asynchrony as observed in this species.

**Maternal resources and female preference**

We found no significant effects of female preference for her mate on either the latency to lay or clutch size. A previous study in budgerigars reported significant effects of mate choice on both traits, but this effect disappeared after the first clutch (Massa et al., 1996). The different results may be due to the fact that the experimental design was not the same. Massa et al. (1996) also used inexperienced birds, but they allowed birds to form pairs during a three month period, and then compared self-selected pairs with randomly paired individuals. The randomly paired birds may have included individuals which had previously established pair bonds with other partners and that were thus artificially divorced. While in our experiment, unpaired females were paired with their preferred and non-preferred partner based on preference scores obtained from an experimental preference test.

Maternal allocation in terms of yolk weight but not egg weight was affected significantly by mate preference and differed between the first and second clutch. The
preference*clutch interaction term was only significant for yolk weight. Our significant results for yolk weight seem to be largely due to the fact that females with the preferred partner produced heavier eggs with heavier yolks only in the second clutch. The differential allocation hypothesis predicts that females should invest more in clutches that are produced when mated with attractive or preferred partners because reproduction is costly and return on investment is higher when mated with attractive males (Burley, 1988; Sheldon, 2000). Producing large and heavy eggs is thought to be costly to the female (Nager, 2006; Vezina and Williams, 2002; Williams, 2005) and hatching from heavier eggs with heavier yolks has been shown to be beneficial for the offspring (Christians, 2002; Krist, 2011; Wagner and Williams, 2007; Williams, 1994). In accordance with the differential allocation hypothesis, experimental studies on several species report that females invest more in clutches of attractive males by increasing egg size and/or mass, although some studies did not find such a relationship (reviewed in: Cucco et al., 2011; Horvathova et al., 2012).

We found that female budgerigars that were paired with their preferred partner laid eggs with yolks that contained more A4 but not T. Females of a variety of species have been found to increase maternal androgen allocation when mated with attractive partners while other studies, mainly in wild populations, did not report such an effect (reviewed in: Kingma et al., 2009). In contrast to maternal investments in terms of egg production (e.g. egg weight, yolk weight), which are thought to be costly to the female (Nager, 2006; Vezina and Williams, 2002; Williams, 2005), there is little evidence to support that females experience direct costs from maternal androgen deposition (Groothuis et al., 2005a; Groothuis and Schwabl, 2008). As maternal androgens impose no direct costs to the female, the differential allocation theory cannot explain why females allocated more androgens to the eggs when they were mated with their preferred partner. Possibly, the females increased the allocation of androgens to stimulate the male to feed more (Groothuis et al., 2005a; Moreno-Rueda, 2007; Müller et al., 2007) as exposure to higher levels of maternal androgens during early development may increase offspring begging behavior (e.g. Eising and Groothuis, 2003; Schwabl, 1996; von Engelhardt et al., 2006). In species in which male attractiveness is negatively correlated with food provisioning, females should thus allocate more androgens to the eggs of attractive males (Moreno-Rueda, 2007). It seems unlikely that this hypothesis can be applied here, as male budgerigars usually provide little direct paternal care during the nestling phase (Stamps et al., 1985), but it could be that begging behavior affects male provisioning indirectly by increased courtship feeding.

Alternatively, even if the deposition of maternal androgens per se is not costly to the
female, there may be an indirect cost due to trade-offs at the level of the offspring (Groothuis et al., 2005a). Chicks may benefit from more maternal androgens because they hatch earlier, grow faster, are more alert and competitive and have higher survival rates (review in: Groothuis et al., 2005a). Nevertheless, several studies indicate that higher levels of T in the yolk may also induce immune suppression, elevated metabolic rate, oxidative stress and parasite susceptibility (Andersson et al., 2004; Cucco et al., 2008; Groothuis et al., 2005b; Müller et al., 2005; Navara et al., 2005; Sandell et al., 2009; Tobler et al., 2007; but see: Navara et al., 2006; Noguera et al., 2011; Rubolini et al., 2006; Tschirren et al., 2005). Such negative effects on the fitness of the offspring may even continue through adult life (Müller et al., 2009; Schwabl et al., 2012). Possibly, we found that females allocate more A4 in clutches produced with preferred males, because the resulting offspring are of higher genetic quality. As higher quality offspring may be better able to cope with the costs of maternal androgens, they may benefit more from the associated positive effects (Gil et al., 2004). This hypothesis may especially apply to species which breed colonially and/or in nest cavities, such as the budgerigar (Brockway, 1964; Wyndham, 1981). In such species, chicks are more likely to encounter parasites and pathogens in the nest (Berkunsky et al., 2005; Brown and Brown, 1986; Tomas et al., 2007, Walls et al., 2012), which may render them more susceptible to the potential immuno-suppressive effects of yolk androgens (Gil et al., 2007; Müller et al., 2004).

Interestingly, we found that females allocated more A4 but not T to the yolk when mated with their preferred partner. Although the yolk contains at least three androgens (T, A4 and 5α-dihydrotestosterone; Schwabl, 1993), so far most research has focused on T, largely neglecting the potential functions of the other androgens in the yolk (Gil et al., 2007). However, the functional roles of different androgens in the yolk may not be the same, as indicated in a number of recent publications (e.g. Eising et al., 2001; Eising and Groothuis, 2003; Hegyi and Schwabl, 2010; Hegyi et al., 2011; Muriel et al., 2013; Tschirren et al., 2014). Moreover, it seems that maternal androgens such as T and A4 are differently influenced by genes and the environment and are under opposing directional selection, which provides further supports for the notion that these two maternal hormones may play different functional roles (for a discussion on this topic, see: Tschirren et al., 2009; 2014). In contrast to T, A4 shows a very low affinity to the androgen receptor, and high levels of A4 are probably less harmful to the embryo. A4 is a metabolite of the ovarian steroid hormone synthesis pathway and can be converted to T by 17β-hydroxysteroid dehydrogenase (Groothuis and Schwabl, 2008; Horton and Tait, 1966). By allocating higher levels of A4 to the yolk, the female may provide the embryo with a source of T, while avoiding potential toxic effects of
high T levels (Bruggeman et al., 2002; Gil et al., 2007; Groothuis and Schwabl, 2002).

Although A4 is thought to impose low immediate costs to the developing embryo, we hypothesize that the eggs produced with preferred males contain higher levels of A4 because the offspring of higher quality males may benefit more from increased T levels. This depends on the conversion of A4 to T by enzymes produced by the embryo in the course of development, needed for its own endogenous T and estrogen production. Differences in the functional significance of different androgens may also explain why we found, similarly to many other avian species, higher levels of A4 than T in budgerigar yolks (Gil et al., 2007). Our findings suggest that the functional role of each androgen in yolk should be considered separately, based on their presence and concentrations in a study species. Many questions regarding the adaptive significance of maternal hormones in the yolk still remain unanswered. Important new insights may come from studies focusing on the physiological mechanisms through which these hormones may affect the offspring (Carere and Balthazart 2007; Pfannkuche et al. 2011; von Engelhardt and Groothuis 2011).

A total of 28% of the females showed a significant preference the first time they were tested and 76% of the females were successful during their second preference test. Similar rates of success have been reported for previous preference experiments (e.g. von Engelhardt et al., 2004). Possibly, many preference tests were not successful because the males of a stimulus set did not differ sufficiently enough to enable the females to discriminate between them. The males of a stimulus set did not differ in body weight, but they differed significantly in UV chroma of the chest, a trait that had been shown to reflect male quality and to be involved in female mate choice (Griggio et al., 2010a, b; Zampiga et al., 2004). This renders it unlikely that the females could not discriminate between the males. It may also be that some females were affected by the social situation (i.e. being housed individually with limited possibilities for social interactions) during the preference tests (Cohen and Wills, 1985). Budgerigars are a highly social species (Brockway, 1964), and although we allowed the birds to have a long habituation period prior to the preference tests, the social situation may have influenced the behavior of some females. If this was the case, females were eventually more used to the experimental conditions during their second preference test, as more females were responsive during the second test. Although the preference test apparatus we used allows for unambiguous and fast measurement of preferences, it is possible that there are limitations as to how accurately female budgerigar preferences can be inferred from our experimental apparatus (Rutstein et al., 2007). We are, however, convinced that our method is reliable as preferences measured in the same species, using similar apparatus, have been shown to be
repeatable between studies (Griggio et al., 2010a; Zampiga et al., 2004) and to be reflected in pairing patterns (Moravec et al., 2006, 2010). This indicates that the preference scores obtained using this method, are a good measure for sexual preferences.

We found that females did not prefer males with higher values for UV chroma of the chest. This result was unexpected as two independent previous studies have found that UV chroma of the chest significantly predicts female choice (Griggio et al., 2010a; Zampiga et al., 2004), but it may be caused by the rather small sample size. Although we did not find a significant effect of chest coloration on female preference, there was a weak trend that females preferred to associate with males that expressed higher values for UV chroma. Furthermore, in contrast to the previous studies, which allowed females to choose between males treated with UV blocker and untreated males (Griggio et al., 2010a; Zampiga et al., 2004), we only measured natural variation in UV chroma. This might explain why the females in our study did not seem to use UV chroma of the chest as a decisive cue during mate preference in all cases. Other male traits, such as the production of warbling song and contact call similarity, have also been found to be important cues in female mate choice in the budgerigar (Moravec et al., 2006, 2010), but these traits could not be considered in this study.

Maternal resources and laying order

Our results show that all six maternal resources measured in this study, varied significantly over the laying order. These measures, however, neither changed linearly with the laying order, nor in interaction with any of the other factors measured. Hence, females did not compensate for the consequences of hatching asynchrony, giving that chicks hatching from larger or heavier eggs with heavier yolks and more yolk androgens would have benefited from hatching earlier, weighing more, growing faster and having higher survival rates (Christians, 2002; Groothuis et al., 2005a; Krist, 2011; Schwabl, 1996; Wagner and Williams, 2007; Williams, 1994). In contrast, we found that in budgerigar clutches earlier-laid eggs were heavier and contained more yolk. We also found that the concentrations of both T and A4 in the yolk are higher in the second egg compared to the first, while being lower again among the last eggs. For other species of parrot similar allocation patterns have been described (Budden and Beissinger, 2005; Kozlowski and Ricklefs, 2010). The functional consequences have as yet to be studied, but it seems unlikely that the observed allocation of maternal resources functions to reduce hatching asynchrony and/or its negative effects on the younger chicks. However, one may also have to consider whether and to what extent these patterns reflect physiological processes during laying (Christians, 2002; Groothuis et al., 2005a;
Interestingly, brood reduction is — despite the allocation patterns we found — uncommon in parrots, and usually the majority of chicks survive until fledging (e.g. Krebs, 1999; Masello and Quillfeldt 2002; Spoon et al., 2006; Stamps et al., 1985; Wyndham, 1981). Similarly, in species of several other avian taxa, maternal allocation does not increase with the laying order although brood reduction is uncommon (Eising et al., 2009; Ellis et al., 2001; Gil et al., 1999; Reed and Vleck, 2001). It may be that in these species, alternative mechanisms are more important in overcoming the competitive disadvantages of later-hatched chicks. These alternative mechanisms may be especially important in the budgerigar, and in other species in which the chicks hatch over a long period of time, because differential maternal allocation is potentially insufficient to compensate for the extreme hatching asynchrony (Muller and Groothuis, 2013; Winkler, 1993).

In the budgerigar, preferential feeding by the female may provide such an alternative mechanism. Newly hatched budgerigar chicks show little begging activity, yet female budgerigars are known to feed the smallest chick first, irrespective of the begging behavior of its siblings (Stamps et al., 1985; our personal observation). This female strategy may diminish the competitive disadvantage of the younger chicks due to the hatching asynchrony, even without affecting the asynchrony itself. Actually, one may wonder why females do not control the degree of hatching asynchrony simply by postponing the onset of incubation if hatching asynchrony were disadvantageous (Groothuis et al., 2005a). This consideration may especially be relevant in cavity-nesting species such as the budgerigar, as in these species early incubation is less likely to be necessary in order to protect the eggs from predation or precipitation (Magrath, 1990; although other factors such as ambient temperature or microbial infection of the egg shell may potentially affect the onset of incubation; Cook et al., 2003; Stoleson and Beissinger, 1999; but see: Walls et al., 2012). In contrast, female budgerigars usually initiate incubation after laying the first or second egg, thereby stimulating asynchronous hatching (Brockway, 1964). This suggests that hatching asynchrony may be adaptive in budgerigars for reasons other than brood reduction. For example, size differences between the chicks due to the hatching asynchrony, may enable the spread of the brood’s peak food demands or may reduce sibling competition if it results in the formation of a stable size hierarchy (Hahn, 1981; Kozlowski and Ricklefs, 2010; Magrath, 1990; Stamps et al., 1985; Wiebe and Bortolotti, 1994). If this is the case, it may not be surprising that the deposition patterns of maternal resources we found in budgerigar clutches do not appear to be unambiguously related to the hatching asynchrony. Whether or not maternal resources in the
egg serve other adaptive functions in the budgerigar remains to be elucidated.

To summarize, we found that female budgerigars when mated with their preferred partner did not initiate egg laying earlier and did not produce larger clutches, but they increased reproductive investment by producing clutches with eggs that contained more yolk and more A4 but not T in the yolk. Interestingly, we did not find evidence that the high chick survival – despite the extreme hatching asynchrony in budgerigars – is due to differential allocation of prenatal resources by the female.
Acknowledgements

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Table 1: Mixed models investigating the effects of female preference on egg weight and yolk weight. Preference, laying order, clutch and their interactions were included as fixed effects and clutch size as a covariate. The degrees of freedom, F-value and P-value are given for the last step where the variable was included in the model. Significant effects (P<0.05) are highlighted in bold.

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<th>df</th>
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Table 2: Mixed models investigating the effects of female preference on the concentrations and the total amounts of both T and A4 in the yolk. Preference, laying order, clutch and their interactions were included as fixed effects and clutch size as a covariate. The degrees of freedom, F-value and P-value are given for the last step where the variable was included in the model. Significant effects (P<0.05) are highlighted in bold.

<table>
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<td>0.13</td>
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Figures Legends

**Figure 1: Diagram of the preference test apparatus used in the experiment.**
The black lines represent the perches. The gray squares represent the food and water containers. Dotted lines represent grated cage walls. The hatched areas indicate the left and right choice area; the open area in the large central part of the cage indicates the no-choice area.

**Figure 2: Female preference based on association score points.** Association score points above and below the 45° line represent respectively females that showed a preference ($P<0.05$) for the male with the higher and the male with the lower value for UV chroma of the chest. Points further from the 45° line indicate a stronger preference. Breeding results according to preference are represented as follows: females that produced a clutch with: both the preferred and the non-preferred partner (open squares), neither the preferred nor the non-preferred partner (black circles), only the preferred partner (open triangles), only the non-preferred partner (gray triangles).

**Figure 3: Maternal allocation according to preference.**
Maternal resource allocation (mean ± SE) to the eggs produced by females paired with the preferred (open bars; $n_{clutch1}=12$; $n_{clutch2}=6$) and non-preferred partner (gray bars; $n_{clutch1}=8$; $n_{clutch2}=10$): A) egg weight, B) yolk weight, C) concentrations of T in the yolk, D) concentrations of A4 in the yolk, E) total amounts of T in the yolk, F) total amounts of A4 in the yolk. The females of each group laid at maximum two clutches and the order of the preference treatment (mated with preferred or non-preferred partner) was reversed for the two groups of females. Four females only produced a first clutch (two with the preferred and two with the non-preferred partner).

**Figure 4: Maternal allocation and clutch size.**
Maternal resources (mean ± SE) in relation to clutch size for the eggs produced by females mated with the preferred (open squares) or non-preferred partner (black squares) for A) yolk weight and egg weight (SE for egg weight are too small to be represented by error bars; range: 0.018 – 0.051), B) concentrations and total amounts of T in the yolk and C) concentrations and total amounts of A4 in the yolk. Clutch size ranged from 3 to 8 eggs. Sample size for females with the preferred and non-preferred partner was respectively: $n_{3eggs}=1:1$, $n_{4eggs}=0:1$, $n_{5eggs}=2:3$, $n_{6eggs}=9:8$, $n_{7eggs}=3:5$, $n_{8eggs}=3:0$. 
**Figure 5: Effects of laying order.**

Variation in maternal resources (mean ± SE) with laying order: A) egg weight (open circles) and yolk weight (black circles), B) concentrations of T in the yolk (black circles) and concentrations of A4 in the yolk (open circles), C) total amounts of T in the yolk (black circles) and total amounts of A4 in the yolk (open circles). Levels not connected by the same letter are significantly different (Tukey adjusted post-hoc tests, $P_a<0.05$).
Figure 2
Figure 3
Figure 4
Figure 5
References


