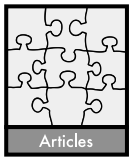


J. Avian Biol. 40: 254–262, 2009
doi: 10.1111/j.1600-048X.2009.04553.x

Received 21 April 2008, accepted 15 July 2008

Do female spotless starlings *Sturnus unicolor* adjust maternal investment according to male attractiveness?

Isabel López-Rull and Diego Gil

I. López-Rull (correspondence) and Diego Gil, Dept. de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, E-28006 Madrid, Spain. Email: isalopez@mncn.csic.es

In birds, female egg allocation patterns have a strong influence in offspring development and differential investment in egg size and composition has been shown to respond to male attractiveness. In this study we experimentally manipulated the perceived attractiveness of male starlings *Sturnus unicolor* by increasing the amount of green material in some nests (a male courtship display in this species). We predicted that, if female investment before laying is related to male attractiveness, experimental females would increase their reproductive investment in response to the addition of plants in their nests when compared to control females. We found that our manipulation caused variations in female reproductive investment in a way that seems to influence offspring quantity but not offspring quality: Females laid larger clutch sizes but not larger eggs when green plant material was added. However, yolk androgens contents were not related to the experimental manipulation. Contrary to expectations, females breeding in experimental nests laid eggs with smaller amounts of eggshell pigments. Interestingly, we found that eggs laid later in the sequence had higher testosterone levels and showed more intense egg colouration than eggs laid earlier in the sequence. These differences at the within-clutch level suggest that selection has favoured compensatory strategies for hatching asynchrony. Alternatively, since nest sabotages by other females are most common at the beginning of laying, this could be seen as female strategy to minimise losses due to nest sabotages. As far as we know, this is the first study to show that an external egg characteristic such as blue-green colouration reflects yolk androgen concentration.

The differential allocation hypothesis (Burley 1988) predicts that selection favours individuals that allocate resources depending on the characteristics of their mate, specifically their attractiveness, since according to sexual selection theory, these traits would be honest indicators of the value of the reproductive attempt. Female reproductive investment can be measured at different stages of the offspring development such as egg formation, incubation and post-natal development (Mousseau and Fox 1998). In birds, the egg provides a sealed environment, which allows no further adjustments of resource investment in the embryo once the egg is laid. Therefore, female allocation patterns during egg formation have a strong influence in offspring development and survival. Examples of this type of differential investment before laying have been found in egg size and composition (e.g yolk androgens, antibodies, carotenoids), which are critical traits influencing fitness in birds and have been demonstrated to be influenced by male attractiveness (Gil et al. 1999, Cunningham and Russell 2000, Saino et al. 2002).

Several male sexual traits other than those displayed in external morphology may act in signalling processes during courtship. For example, in many avian groups, males carry specialized materials into the nests, such as plants, stones or prominent decorations, that provide reliable signals of mating status or genetic quality (Borgia and Gore 1986, Moreno et al. 1994, Gwinner 1997, Soler et al. 1998, Fargallo et al. 2001, Duffy and Ball 2002, Brouwer and Komdeur 2004, Polo et al. 2004, Veiga et al. 2006). Males of the European *Sturnus vulgaris* and the spotless starling *Sturnus unicolor* carry green material into their nests before the laying period (Gwinner 1997, Veiga et al. 2006), and it has been recently proposed that this display has a function on mate attraction that may convey important information on male quality to females (Gwinner et al. 2000, Brouwer and Komdeur 2004, Polo et al. 2004, Veiga et al. 2006).

In this study we experimentally manipulated the perceived attractiveness of male starlings by increasing the amount of green material in nests. If female investment before laying is related to male attractiveness, we would expect experimental females to increase their reproductive investment in response to the addition of fresh plants in their nests when compared with control females. The predictions that we test are the following: 1) Females mated with more attractive males will lay larger clutch sizes or larger eggs than females mated with less attractive males. Egg production is expensive in terms of both energy and nutrient requirements and it has been demonstrated that experimentally increased egg production can reduce the capacity of the parents to rear the young thus causing

negative effects on fitness. For this reason a trade-off between clutch size and egg size is expected. It has been demonstrated that in some bird species females paired with more attractive males lay more eggs than females paired with less attractive males (Petrie and Williams 1993, Schwabl et al. 1997). Because high quality females lay larger clutches than lower quality females (Drent and Daan 1980, van Noordwijk and de Jong 1986, Rowe et al. 1994, Christians et al. 2001 for starlings), and larger clutches generally fledge more chicks and produce more recruits (Price and Liou 1989, Rowe et al. 1994), clutch size can be used as an indicator of maternal reproductive investment. In addition, more subtle forms of differential allocation could also be occurring and females could alter their reproductive effort in ways that influence offspring quality instead of offspring quantity, for example by laying larger eggs when paired with more attractive males (Cunningham and Russell 2000). 2) Females mated with more attractive males will transfer higher amounts of androgens to their eggs than females mated with less attractive males. Variations in androgen concentration between eggs have been shown to contribute to differences in offspring phenotype and fitness and thus adaptive patterns of yolk androgen deposition such as differential allocation in relation to male quality have been reported (Gil et al. 1999, Tanvez et al. 2004). Although there is experimental evidence that high levels of yolk testosterone (T) beneficially influence the physiology and behaviour of nestlings (Lipar and Ketterson 2000, Eising et al. 2001, Eising and Groothuis 2003, Pilz and Smith 2004), elevated yolk T was also found to suppress immune functions (Duffy and Ball 2002, Eens et al., 2000, Müller et al., 2005, Uller and Olsson 2003), and negative effects or no effects of increased yolk androgens on phenotype or viability have also been reported (Navara et al., 2005; Sockman and Schwabl, 2000; Andersson et al., 2004, Rubolini et al., 2006a, 2006b, Sockman and Schwabl, 2000, Uller et al. 2005), suggesting that these constraints may limit the amount of hormones that females may allocate to eggs or that offspring may tolerate (Gil, 2003, 2008). Because females have been shown to increase yolk androgen levels when mated to attractive males (Gil et al. 1999, Gil et al. 2004, Tanvez et al. 2004), it is possible that benefits of yolk androgens are limited to offspring with certain phenotypic or genotypic quality. 3) Females mated with more attractive males will lay more colourful eggs than females mated with less attractive males. It has been proposed that females colour their eggs using costly pigments in order to signal their quality to their mates and thus induce a higher allocation of paternal care (Moreno and Osorno 2003). This hypothesis is based on the antioxidant properties of the pigment biliverdin (Kaur et al. 2003) which causes the blue–green colouration in avian eggs (Miksik et al. 1996), leading to the possibility that eggshells may indicate female antioxidant capacity. Egg colour has been shown to predict concentration of biliverdin eggshell (Moreno et al. 2006, López-Rull et al. 2008), the quality of the eggs themselves (Morales et al. 2006) and the fitness of the resulting offspring (Morales et al. 2006). Therefore, blue–green colouration may represent a form of costly maternal investment, and we expect females mated to attractive males to lay high quality eggs with more intense blue-green colouration. 4) Females mated with more attractive males will present higher plasma testosterone levels than females mated with less attractive males. High levels of female competition for males and nest sites in this species result in frequent female fights and clutch sabotages, sometimes leading to females being killed in their nests (López-Rull and Gil, unpubl. data). Since high testosterone levels may help females to increase their resource holding (Veiga and Polo 2008), we expect experimental females to present higher testosterone levels than control females. In addition, we will evaluate if endocrine state of females may influence maternal investment in terms of yolk androgen deposition.

Methods

General procedures

The experiment was conducted in a large colony of spotless starlings in Soto del Real, Madrid during the breeding season of 2006. The study area consists of a deciduous woodland of oak *Quercus rotundifolia* and ash *Fraxinus angustifolius*, in which nest boxes are distributed since 2001 and the population has been under study since then.

The facultative polygynous spotless starling is a medium-sized relatively long-lived species, which is closely related to the European starling *Sturnus vulgaris*. It is commonly double brooded and during courtship male starlings incorporate green material into their nests (in our population mainly *Lavandula stoechas*, *Santolina rosmarinifolia*, *Geranium robertianum* and *Lamium purpureum*) until the first egg appears (Veiga et al. 2006). Incubation usually starts before the last egg is laid and the major part of nestling feeding is performed by the female (Veiga 2002).

The experimental manipulation was done following procedures successfully used before by Polo et al. (2004, 2006). In first clutches, we manipulated the amount of green plants in the nest by visiting nest boxes and randomly assigning them to either the experimental ($n = 20$) or control group ($n = 20$) ensuring the same distribution of dates among treatments (laying dates did not differ between treatments: $F_{1,33} = 1.73$, $P = 0.20$).

Approximately a month before egg laying started nest boxes were visited daily and about 15 g of fresh plants (mainly *Lavandula stoechas*, *Santolina rosmarinifolia*, *Geranium robertianum* and *Lamium purpureum*) were incorporated into the experimental nest boxes. The amount of fresh plants was similar to the maximum amount recorded in nests of high quality males, so the stimulus created by our experimental manipulation was within the range of natural levels experienced by the starlings in the wild. No green material was incorporated in control nests, but we spent the same time visiting them, so that the degree of disturbance was similar for both groups. Spotless starling females generally remove the green nesting material, both the experimental material added by researchers and those incorporated by males.

Manipulations were discontinued when the first egg appeared in each nest but daily visits continued in both groups to determine laying order. Eggs were marked with a non-toxic marker as they were laid and measurements of egg colour, length and width were taken. Egg colour was

measured using an Avantes spectrophotometer (AvaSpec-2048FT), which covered reflectance in the avian visible spectrum (300–700 nm; Endler and Mielke 2005). The data output consisted of 40 reflectance values in steps of 10 nm. A reference calibration within a standard white was taken prior to the colour measurements according to the apparatus specifications.

Before incubation started, approximately 25 mg of yolk was taken from every egg in the clutch. Biopsies were done by inserting the needle of a 25 gauge winged infusion set into the yolk. In order to ascertain that the tip of the needle penetrated the yolk, eggs were candled from beneath with a small torch. New needles were used in every new egg. After sample removal the hole was sealed with a tiny strip of flexible wound dressing (Opsite, Smith and Nephew). Eggs were maintained at ambient temperature during the biopsies and returned to the nest within 10–20 min so that embryo development could continue. Yolk samples were individually weighted and homogenized in 1 ml of distilled water and frozen at -70°C for future androgen analysis. Four days after incubation started females were captured in their nests, biometric data were recorded and a blood sample was taken within 10 min after capture. Samples were kept on ice in the field for 3–8 h before arrival to the laboratory. Plasma was then separated by centrifugation and kept at -40°C until assaying. In order to estimate female quality, three feathers were randomly collected from the central region of the throat from each bird. Removal of feathers was performed by pulling them near the base. Feathers were kept in plastic bags until length measurements were performed with a digital calliper to the nearest 0.01 mm. All clutches were collected for another study.

Hormone analyses

Plasma and yolk steroids were extracted by adding 10X of diethyl ether to the sample, vortexing for 15 min and centrifuging for 10 min (4°C, $2000 \times$RPM). The ether phase was decanted after snap-freezing the tube in an alcohol bath at -30°C. This procedure was repeated a second time and both ether phases were combined in a single tube, and evaporated to dryness. In order to remove further proteins, the dried extract of yolk samples was redissolved in 1 ml of 90% ethanol, kept at 4°C for 12 h and centrifuged for 10 min (4°C, $2000 \times$RPM). Supernatants were dried under a stream of nitrogen, and the dried extract was redissolved in 300–400 μl of steroid free serum (DRG Labs) depending on the amount of yolk used in each sample. Plasma testosterone samples (200 μl) were resuspended in 400 μl of assay buffer (Cayman Chemical).

Yolk concentrations of testosterone (T) and androstenedione (A4) were determined by two different ELISA kits highly specific for each hormone (DSL Labs in the case of T and DRG Labs for A4). According to the manufacturers, cross reactivity of the A4 antibody was less than 1% for all hormones tested and cross reactivity of the T antibody was less than 1% for all hormones tested except for 5α DHT which was 6.6%. Assays were performed according to manufacturer's instructions. Samples were analyzed in duplicate with respect to a standard curve. For T the intra-assay coefficient of variation was 7.25% and the inter assay coefficient of variation was 7.71%. For A4 the intra-assay coefficient of variation was 6.62% and the inter-assay coefficient of variation was 6.03%.

Plasma testosterone was analyzed using a commercially available ELISA testosterone kit (Cayman Chemical). Samples were analyzed in duplicate with respect to a standard curve. The within-assay coefficient of variation was 10.2%. A set of identical internal standards was run in each assay. The inter-assay variation, after correction by means of linear regressions of these standards, was 7.3%. The antibody used in the kit is highly specific for testosterone, but has some cross-reactivity with other androgens (5α-DHT: 27.4%; A4: 3.7%).

Data analyses

Female quality was estimated by calculating the mean length of the 3 feathers collected. Female throat feather length, is an age dependent trait, highly repeatable within females and positively related to reproductive success in this species (Lopez-Rull et al. 2007). Differences in female condition were tested using a linear model including in the analyses body mass as dependent variable, tarsus length as independent covariate and treatment as a fixed factor (López-Rull et al. 2008).

One way ANOVAs were used to analyze differences in female quality, female testosterone, laying dates and clutch sizes between treatments. Out of 35 nests with eggs, we were able to catch 19 control females and 16 experimental females. Plasma testosterone measures are available for 12 control females and 8 experimental females.

Egg volume was calculated by the formula: $0.45 \times \text{length} \times \text{width}^2$ (Worth 1940). Egg colour was measured in 82 eggs belonging to 27 clutches (16 control clutches and 11 experimental clutches). Egg colour was measured by means of Principal Component Analysis (PCA). This method reduces a large number of correlated variables into a few orthogonal variables that summarize most of the variation (Cuthill et al. 1999, Cherry and Bennett 2001). In the PCA, the first two principal components explain together 97.81% of the variance in the spectra. The first principal component (PC1) describes the variance in mean reflectance, it is flat throughout the spectrum of wavelengths and consequently represents achromatic brightness (87.20% of the variance; Eigenvalue = 35.75; Fig. 1). The second principal component (PC2) shows variation in spectral shape thus measuring aspects of the egg's chromatic variation such as hue and saturation (10.61% of the variance; Eigenvalue = 4.35; Fig. 1). PC1 scores are negatively associated with the amount of biliverdin present in the egg (López-Rull et al. 2008).

To compare egg volume, egg colour and yolk androgen deposition between treatments we used Variance Component Models including the following explanatory variables and their interaction terms: nest as a random factor, treatment as a fixed factor and female testosterone and laying sequence as covariates. Non-significant terms were removed from each model ($P < 0.05$). Variables were transformed when necessary to approach normal distributions (logarithmic transformation for egg volume, yolk androgens and female testosterone; after transformations

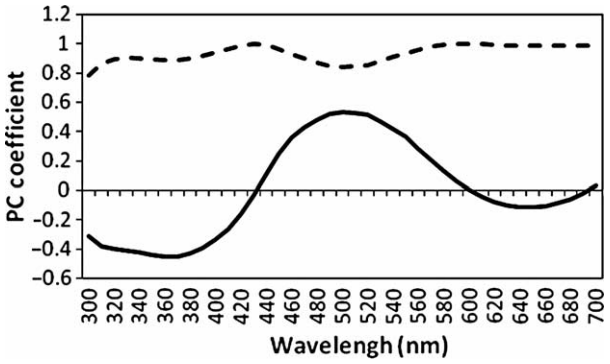


Figure 1. Principal components as a function of wavelength. PC1 (dotted line) is flat and positive all over the spectrum and consequently represents colour brightness, whereas PC2 (black line) shows variation in spectral shape thus measuring aspects of the egg's chromatic variation such as hue and saturation.

variables were normally distributed; in all Shapiro–Wilk tests for normality $P < 0.1$). Statistical analyses were performed with SPSS v.11.5 and STATISTICA.

Results

Female quality, condition and testosterone levels

Neither throat feather length nor body condition differed between experimental and control groups, indicating that female quality and condition were randomly distributed between treatments (feathers: $F_{1,24} = 0.1$, $P = 0.94$; body condition: $F_{2,22} = 3.02$, $P = 0.098$). The experimental addition of green plants increased female testosterone levels in incubating females ($F_{1,18} = 6.54$, $P = 0.02$; Fig. 2). We found an effect of the treatment on the number of eggs laid in experimental and control females ($F_{1,33} = 8.03$, $P = 0.008$). On average, females in the experimental group laid larger clutches than control females (experimental: 4.94 ± 0.68 eggs, $n = 16$; control: 4.10 ± 0.99 eggs, $n = 19$; Fig. 3).

Because intraspecific nest parasitism during the laying period is common in this species (Calvo et al. 2000), it is possible that this result could be due to experimental nests (nest with a more attractive male) being for some reason more vulnerable to parasitism than control nests. We tested this possibility by analysing whether within-clutch variances in egg colour or volume were different between treatments. We found no differences in the within-clutch coefficient of variance in egg colour or volume between experimental and control females (PC1: $F_{1,24} = 0.42$, $P = 0.52$; PC2: $F_{1,24} = 0.07$, $P = 0.79$; log volume: $F_{1,33} = 0.05$, $P = 0.82$). This suggests that brood parasitism rates were not different between experimental treatments. Variation in egg volume was far greater between clutches than within clutches ($F_{1,33} = 13.09$, $P < 0.001$), and declined with the laying sequence ($F_{1,33} = 12.53$, $P < 0.001$). However, egg size did not differ between treatments (log volume: experimental: $3.78 \pm 0.04 mm^3$, $n = 16$; control: 3.79 ± 0.03 mm^3, $n = 19$; $F_{1,\ 33} = 1.56$, $P = 0.22$).

Yolk androgens and egg colour

Significant inter-nest variation was found in yolk androgen deposition (logA4: $F_{1,33} = 1.90$, $P < 0.008$; logT: $F_{1,\ 33} = 1.60$, $P = 0.04$). Both T and A4 were associated to laying order but in different ways: while T increased with laying order ($F_{1,33} = 13.72$, $P = 0.003$), A4 decreased ($F_{1,33} = 7.17$, $P = 0.008$; Fig. 4). No differences between treatments were found (logT: experimental: 0.88 ± 0.15 pg/ml, $n = 16$; control: 0.93 ± 0.18 pg/ml, $n = 19$; $F_{1,33} = 1.43$, $P = 0.24$;

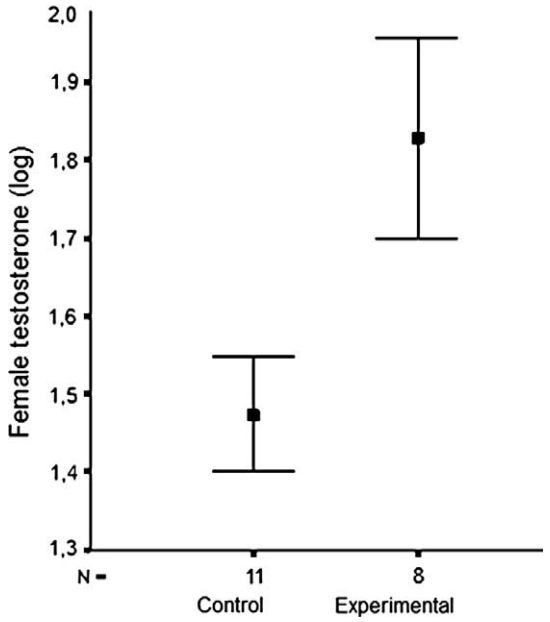


Figure 2. Female testosterone levels (mean ± SD) in experimental and control females.

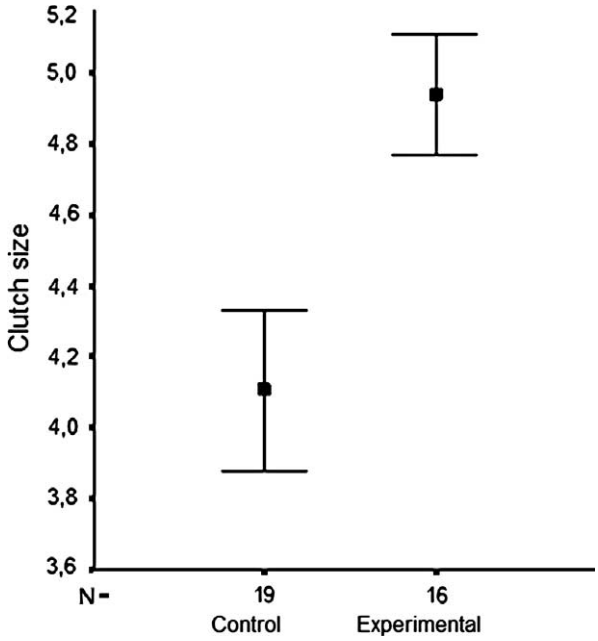
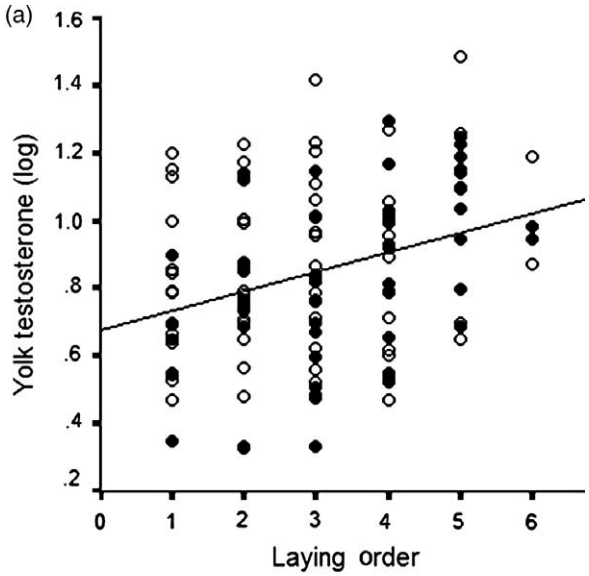


Figure 3. Clutch sizes (mean ± SD) of experimental and control females.



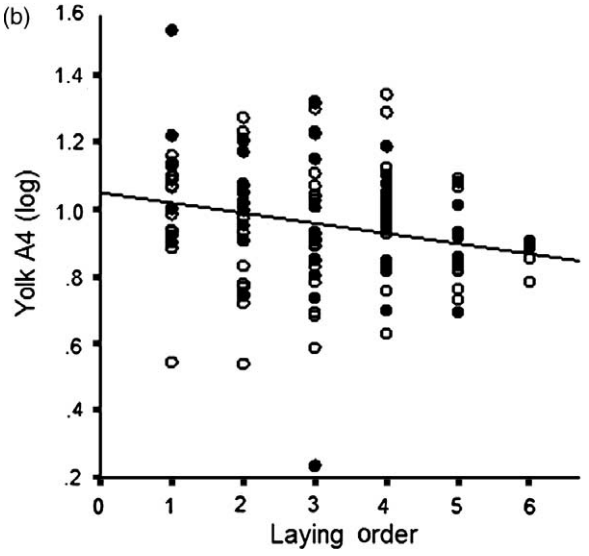


Figure 4. Yolk androgen deposition in relation to laying order. No differences were found between experimental (filled circles), and control (empty circles) females neither in testosterone (2a), nor in A4 (2b).

logA4: experimental: 0.99 ± 0.09 pg/ml, $n = 16$; control: 0.96 ± 0.14 pg/ml, $n = 19$; $F_{1,33} = 0.82$, $P = 0.37$). Maternal testosterone was not related to yolk testosterone (Pearson's $= -0.37$, $P = 0.10$, $n = 20$), or yolk A4 (Pearson's $= -0.20$, $P = 0.39$, $n = 20$).

Clutch size and egg volume

Egg colour as measured by PC1 and PC2 showed more variation between nests than within nests (PC1: $F_{1,25} = 6.75$, $P < 0.001$; PC2: $F_{1,25} = 5.11$, $P < 0.001$). We found that in both treatments eggs laid later in the laying sequence expressed lower PC1 scores than eggs laid earlier in the laying sequence ($F_{1,25} = 10.53$, $P = 0.002$; Fig. 5). When compared to control females, experimental females had higher PC1 scores (experimental: 0.49 ± 0.85, $n = 11$; control: -0.34 ± 0.95, $n = 16$; $F_{1,25} = 6.08$, $P = 0.02$). PC2 showed no association with laying order ($F_{1,25} = 0.02$, $P = 0.89$) and did not differ between treatments (experimental: -0.28 ± 1.15, $n = 11$; control: 0.10 ± 0.89, $n = 16$; $F_{1,25} = 0.76$, $P = 0.39$).

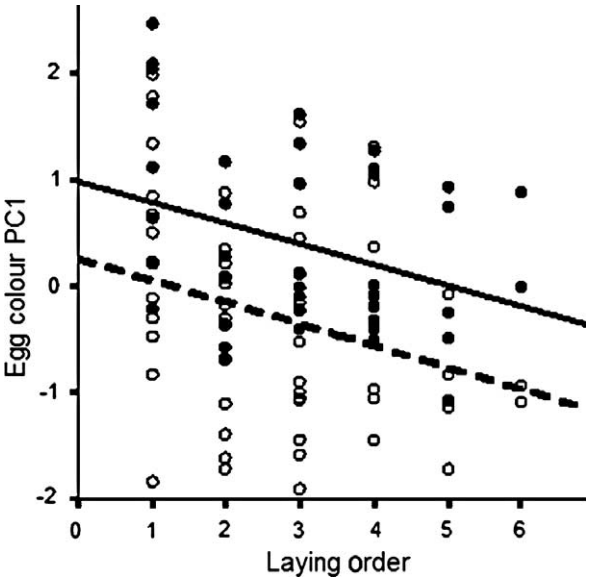


Figure 5. Egg colour PC1 in relation to laying order in experimental (fulfilled circles, continuous line), and control (empty circles, dotted line) females.

Because both yolk androgens and egg colour varied with laying sequence we tested for an association between them, finding that PC1 tended to be negatively related with the amount of testosterone in the yolk ($F_{1,25} = 3.45$, $P = 0.07$). In order to distinguish whether this association was due to an effect of the laying sequence within clutches or an effect of differences between females, we related mean clutch egg colour to mean yolk testosterone levels finding a significant

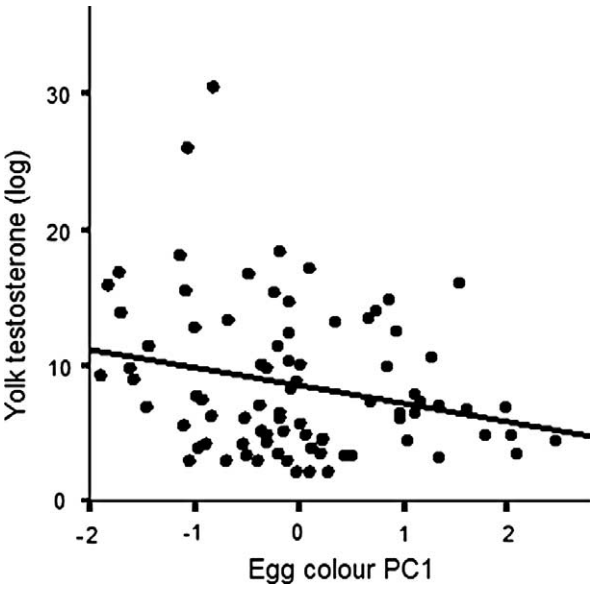


Figure 6. Mean yolk testosterone in relation to mean egg colour PC1.

negative association between them (PC1: $F_{1,25} = 4.88$, $P = 0.037$, $r = 0.40$; Fig. 6).

Discussion

Optimal investment theory predicts that females mated with high quality males should invest more in their offspring than females mated with low quality males. To test this hypothesis we experimentally manipulated male attractiveness by increasing the amount of green material in their nests and recorded female reproductive investment in several reproductive traits.

Variation in female quality and condition was equally distributed between treatments, indicating that the differences that we found cannot be accounted for variation in female phenotype. Our experimental manipulation was successful at inducing differences in maternal investment. We tested whether levels of investment were higher in females mated with more attractive males and found that experimental females alter their reproductive investment in relation to the number, but not quality, of offspring. Females laid larger clutch sizes when green plant material was added but no differences in egg size or yolk androgen concentration were found between treatments. Although increased clutch or brood size may reduce offspring quality through increasing sibling competition and reducing food availability per nestling, in our study population reproductive success is related to clutch size: larger clutches fledge more nestlings than smaller clutches (unpubl. data). Thus, the fact that experimental females produce larger clutches is in agreement with the differential allocation hypothesis. Similarly, in a previous study in the common starling, Pilz et al. (2003) found that clutch size but not egg size was a good indicator of female investment. Although we found significant heterogeneity of yolk androgen levels between clutches, yolk androgen concentration was not related to male attractiveness. Furthermore, although female endocrine state could affect maternal investment, we found no relation between maternal testosterone and yolk androgens. This result is probably due to the fact that female testosterone was sampled during incubation. A previous study (Mazuc et al. 2003) also found a lack of relationship between androgen levels in females and eggs after clutch completion. Additionally, since high levels of yolk androgens may induce costs for the offspring, selection is expected to have favoured mechanism by which females could somehow ''control'' their androgen transfer to the eggs. Such a mechanism would be particularly important in species with strong female-female competition whereas female testosterone levels are likely to increase in order to maintain a territory and a mate, as is occurs in the spotless starling.

If blue–green colouration represents a form of costly maternal investment, we would expect females to vary it according to the attractiveness of their mate. We found that variation in egg colour was greater between clutches than within clutches, however the direction of the relationship was contrary to our predictions, and females from the experimental group showed higher PC1 scores than females from the control group. Because the amount of pigmentation in the eggshell is negatively associated with PC1 (Moreno et al. 2006, López-Rull et al. 2008), this result suggest that females paired with more attractive males lay eggs with smaller amounts of egg pigments. Since egg colouration has been proposed to serve as a post-mating signal of female and egg quality (Moreno and Osorno 2003, Morales et al. 2006) this difference is contrary to expectations. Blue-green eggs are coloured by the pigment biliverdin (Kennedy and Vevers 1976) that is deposited on the shell by the mother's shell gland (Baird et al. 1975). Biliverdin and its reduction product, bilirubin, are potent antioxidants (Stocker et al. 1987), so allocating these chemicals for colouring eggs may be costly for females, particularly when they face an important oxidative stress due to increased testosterone (as a consequence of experimentally adding green material to their nests) and egg-laying period (when high levels of circulating progesterone can induce oxidative stress; von Schantz et al. 1999). A possible explanation to our findings is that our treatment would have increased physiological stress levels, thus leading experimental females to require biliverdin to fight their own oxidative stress instead of deposition in their eggs. Besides our own study, several lines of evidence support this possibility. First, previous research has shown that an increase of the amount of green material in this species results in an increase in female testosterone circulating levels (Polo unpubl. data), which would lead to higher oxidative stress. Second, experimental nests may attract higher numbers of floating females, thus increasing intra-sexual competition between females, which is very high in this species. Under these circumstances it is possible then that females breeding in green-supplemented nests may have used biliverdin to fight the elevated oxidative stress caused by high testosterone levels, thus resulting in a reduction of pigment availability for eggs.

Egg colouration was related to the amount of yolk testosterone: eggs of clutches of mean high yolk testosterone levels were less bright (PC1) than eggs from clutches of mean low yolk testosterone, and thus should contained higher levels of biliverdin. This suggests that males can obtain an estimation of egg androgen contents through egg coloration, in addition to those cues related to female quality or condition (Gil et al. 2004, 2006). We expect this information to be particularly relevant in polygynous species, such as starlings, where male parental contribution is traded off against mate attraction, and where females gain from inducing higher levels of parental care via egg colouration (Moreno et al. 2006, Soler et al. 2007). Previous research has shown that male parental care is higher for nests with a predicted higher reproductive success (Komdeur et al. 2002).

An interesting pattern is the relation between egg colour and yolk T and the fact that both vary with laying order: eggs laid later in the sequence had higher T levels and were more colourful (i.e. higher biliverdin levels). In several bird species, including the common starling, there is evidence that androgens increase with laying order (Eising et al. 2001, Groothuis and Schwabl 2002, Pilz et al. 2003), although in other species the pattern is inverted (e.g. Gil et al. 1999). These within-clutch androgen distributions are particularly relevant to the hypothesis that yolk androgens modulate competitive asymmetries resulting from hatching asynchrony. It is also possible that increased pigmentation

may be a maternal strategy of allocating higher resources to eggs laid later in the laying sequence. Egg colouration can decrease (Moreno et al. 2005, Krist and Grim 2007), or increase with laying order (Siefferman 2006), depending on the species, and such pattern may function to facilitate survival of the nestlings hatched from those eggs. In addition, it has been reported in the pied flycatcher (Morales et al. 2006) that the intensity of blue-green egg colouration is positively related to maternal antibodies in the yolk and to offspring fledging success. In the spotless starling there is a moderate hatching asynchrony (interval of 1–2 d) so it is possible that this form of investment (higher T and biliverdin content) may help compensate asymmetries between siblings.

Another possibility to explain this within-clutch androgen distribution is related to intra-sexual competition. In the spotless starling, antagonistic encounters between females and nest sabotages are frequent before and during laying, as a result of female competition for access to males. Since the risk of nest sabotages is higher at the beginning of the laying period, when females spend less time attending their nests, it is possibly that a lower egg investment at the beginning of laying may reduce the cost of losses due to clutch sabotages.

A special case that may influence female investment and was not considered in our experiment is the brood sex ratio. Although it remains unclear how the female is able to modulate the process of chromosomal sex determination (Pike and Petrie 2003) there is empirical evidence showing that female may adjust offspring sex ratio according not only to her own phenotype (e.g. endocrine state) but also in response to individual differences in male sexual traits (Ellegren et al. 1996, Sheldon et al. 1999). In the spotless starling two recent studies found that the primary sex ratio was biased to males either because an experimental enhancement of the green plants into the nest (Polo et al. 2004) or by an experimental manipulation of female circulating testosterone levels (Veiga et al. 2004). Therefore sex ratio should be considered when evaluating female egg investment in this species, especially if the experimental manipulation increases female circulating levels of testosterone.

In summary, manipulating the amount of green material carried by the male in the spotless starling caused variations in female reproductive investment: experimental females alter their investment in ways that seem to influence offspring quantity but not offspring quality. Our results suggest that selection has favoured an increased allocation of testosterone and egg colouration within the laying sequence possibly as a compensatory strategy for hatching asynchrony or as a female strategy for minimising losses due to intra-female sexual competition. As far as we know, this is the first study to show that an external egg characteristic such as blue-green colouration reflects yolk androgen concentration.

Acknowledgements – We thank M. Gil, J. Fernández and A. González for helping at the field. IL was supported by PhD grants from CONACYT (México). Research was funded by a grant (BOS2002-00105) and a Ramon y Cajal fellowship from the Ministerio de Educación y Ciencia (Spain) to DG. Permission to work in the study area was granted by the Ayuntamiento de Soto del Real and the Consejería de Medio Ambiente of the Comunidad Autónoma de Madrid. This manuscript is a contribution of the field station "El Ventorrillo".

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