ethology international journal of behavioural biology

Ethology

Experimental Addition of Green Plants to the Nest Increases Testosterone Levels in Female Spotless Starlings

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Received: September 17, 2009
Initial acceptance: October 27, 2009
Final acceptance: October 27, 2009
(G. Beckers)

doi: 10.1111/j.1439-0310.2009.01724.x

Abstract

Multiple male traits and displays may act in signalling sexually selected processes during courtship. Spotless starling males (*Sturnus unicolor*) carry green plants into their nests before egg laying, and recent studies have shown that this behaviour is related to female breeding decisions and the production of male-biased broods. Although the functional implications of this effect on females are not yet clear, data suggest that it could be mediated by female circulating hormones. Additionally, females may show higher androgen levels as a consequence of the increased female–female competition generated by the increase in male attractiveness. We tested this hypothesis using the same manipulation of green nesting material that has been previously shown to result in an increase of male attractiveness in male spotless starlings. We found that females in experimental nests increased their circulating testosterone levels during the laying period. In addition, there was an increase of social interferences in the experimental nests because of the addition of green plants. We hypothesise that testosterone may allow females to maintain their mating status when competing with other females for the preferred males. Addition of green plants also increased the variance in the levels of circulating testosterone, suggesting plasticity between females in their response to the manipulation. We propose that there is a functional link between high testosterone levels, male-biased sex ratios and female resource-holding potential in intra-sexual competition in this species.

Several male specific characteristics other than morphological traits may act in sexually selected signalling processes during courtship (e.g. Borgia & Gore 1986; Moreno et al. 1994; Duffy & Ball 2002; Polo et al. 2004; Veiga & Polo 2005). For instance, males of the European (*Sturnus vulgaris*) and the spotless starling carry green material to their nests during nest building and before the onset of laying (Gwinner 1997; Veiga et al. 2006). The function of this male behaviour has long puzzled biologists, and early research was directed to examine the possible anti-parasitic effects of these plants (Clark 1991; Fauth et al. 1991; Hansell 2000; Gwinner & Berger 2005). However, recent studies have shown that this display has a function in mate attraction and may convey important information on male quality to the females (Gwinner et al. 2000; Brouwer & Komdeur 2004; Polo et al. 2004; Polo & Veiga 2006).

Avian females may adjust offspring sex ratio in response to individual differences in male sexual traits or displays (Ellegren et al. 1996; Sheldon et al. 1999; Polo et al. 2004), and in relation to differences

in female age (Veiga et al. 2008). Our own work has shown that female spotless starlings increase the proportion of males in their broods in response to an experimental increase in the amount of green plants in their nests (Polo et al. 2004; but see Veiga et al. 2008). As the amount of green material carried by male starlings is related to their circulating levels of testosterone, body condition and territorial status (De Ridder et al. 2000; Pinxten et al. 2002; Veiga et al. 2002, 2006), this behaviour may constitute a sexually selected display in this species.

A recent study has underlined a link between high female testosterone levels, nest-box holding potential and male-biased sex ratio in the spotless starling (Veiga et al. 2004). It is thus conceivable that the mechanism of sex ratio adjustment may be mediated by levels of circulating androgens in females (Veiga et al. 2004). Similarly, increased levels of circulating testosterone in breeding females may mediate the association between male addition of green plants and male-biased sex ratios.

Intra-sexual competition between females for preferred males or nest-sites is strong in the spotless starling. In birds, aggressive behaviour appears to be under hormonal control and in many species androgens are involved in mediating aggression and territorial defence ('the challenge hypothesis': Harding 1983; Wingfield 1994; see McGlothlin et al. 2007 for a testosterone-mediated trade-off between territorial aggression and parental care in males). This hypothesis would thus predict that females may enhance their levels of circulating androgens as a consequence of the increase in female–female competition generated by the presence of more attractive males (Wingfield et al. 1990; Goymann et al. 2007).

The aim of this study was to detect whether females increase their androgen levels as a consequence of the manipulation of green material in nests. We experimentally increased the amount of green plants in the nests of spotless starlings, hence simulating the presence of high quality males, following the same protocol as in our previous study (see Polo et al. 2004; Veiga et al. 2008; for details), and we measured the effect of this manipulation on female circulating testosterone levels during laying. As females are expected to increase their ability in intra-sexual competition for the preferred males in relation to their level of testosterone (Searcy 1988), we predict that females should increase their levels of circulating androgens when receiving high amounts of green plants into their nests. A secondary purpose of this work was to identify a proximate mechanism involved in the adjustment of circulating testosterone levels of females. We predict that the greenery should increase the amount of nesting interferences caused by the increase in aggressiveness between females during nest construction, laying and incubation, leading to an increase in female androgen levels.

Methods

Study Area and Experimental Procedures

The spotless starling is a medium-sized (90 g), relatively long-lived (up to 8 yrs), commonly double brooded and facultatively polygynous passerine (Moreno et al. 1999; Veiga et al. 2001, 2002), closely related to the European starling. The experiment was conducted in a breeding colony of spotless starlings in Madrid province (central Spain) between the end of March and the end of May in three consecutive years (2004–2006). The habitat consists of pastureland with scattered trees (ashes *Fraxinus angustifolia*, oaks *Quercus pyrenaica* and holm oaks *Quercus rotundifolia*). A total of 54 nest-boxes were placed in the colony during 1997. Nest-boxes were spaced ca. 30–60 m apart. All breeding birds were marked with an aluminium band and a unique combination of coloured plastic bands for individual identification.

During courtship, males bring green plants to their nest. Male starlings begin to incorporate green material into their nests 8–10 d before the onset of laying and cease this activity once the first egg appears (Gwinner 1997; Brouwer & Komdeur 2004; Veiga et al. 2006). The manipulation consisted of daily green plant additions in the experimental nest-boxes from 10 d before the expected date of clutch initiation in the colony until the first egg appeared in each nest. Laying date is highly synchronous in this species so we can use information on nest construction (mainly by the females) to predict the start of laying in the colony and thus the date for the beginning of the green plants additions in the experimental nest-boxes. We were successful at predicting the start of laying because we added plants during 10–12 d in most experimental nests (range 6–14 d; see below). We randomly allocated 27 boxes to the experimental group, whereas another 27 remained as controls. The assignation of each nest-box to control and experimental groups varied in the 3 yrs of the study. We spent less than 5 min daily per nest-box to add green plants in experimental boxes, to inspect the plants added by males in control boxes and to look for nesting interferences (see below) in

both control and experimental boxes. Plants added by males were not removed from control nests. We chose a fixed daily itinerary that alternated time of manipulation/inspection between experimental and control boxes. Thus, time and duration of human disturbance were similar in control and experimental nest-boxes. As we randomly allocated the boxes to the two groups, we do not expect differences between groups in the level of polygyny prior to the beginning of the manipulation.

We followed Veiga et al. (2006) for quantifying the reproductive experience of the females. Females breeding for the first time and those that had bred only once before the year of the study were classified as inexperienced, and the rest as experienced females. This variable was used as a categorical factor in the analyses. Our manipulation took place after the nest-boxes had been owned and defended by a male. However, changes between females in nest-box ownership, mostly due to female–female competition, can occur during our nest manipulation period.

The amount and disposition of fresh plants in nests were similar to the maximum amount recorded in nests of high quality polygynous males, and thus the stimulus created by our experimental manipulation was within the range of natural levels experienced by the starlings in the wild (Veiga et al. 2006). In the experimental nests, we placed shredded fresh plants daily (approx. 10–20 g of fresh plants). The green plants used belonged to the species added naturally by male starlings of these populations (mixed amounts of *Lavandula stoechas*, *Santolina rosmarinifolia*, *Geranium robertianum* and *Lamium purpureum*, and leaves of *Fraxinus angustifolia* and *Quercus pyrenaica*; see Polo et al. 2004; Veiga et al. 2008). We placed these shredded plants covering completely the nest each morning until the first egg appeared. Starling females generally removed the green nesting material, both the experimental plants added by the researchers and those put into the nest by males, probably as a way of stopping new females from being attracted to the nest. We quantified the amount of greenery carried by males in the control nest-boxes by using the same five categories described in Veiga et al. (2006). The categories were as follow: 0 (absence of green plants fragments into the nest); 1 (a single fragment smaller than 5 cm or two fragments lesser than 2 cm); 2 (two or three fragments between 2 and 5 cm or three to five fragments lesser than 2 cm. Occasionally a single fragment larger than 5 cm); 3 (four or five fragments between 2 and 5 cm or six to eight fragments lesser than 2 cm. Occasionally two or three fragments larger than 5 cm); and 4 (more than five fragments between 2 and 5 cm or more than eight fragments lesser than 2 cm. Occasionally more than three fragments larger than 5 cm). Mean level of green material in control boxes (0.65 ± 0.12) was clearly much lower than in the experimental group (level 4).

Ethical Note

We use custom-made traps fitted into the nest-boxes to captures breeding birds in the colony. Most unringed breeders (less than 25% of total breeders in the colony) were captured and ringed at the end of March. Chicks were marked with an aluminium ring when 5- to 6–d old. Thus, as adult survival and natal philopatry are high in this species, most individuals were already ringed in their first capture as breeders.

Spotless starlings are accustomed to human presence, as they normally breed in buildings, and the study colony has been monitored since 1997 with a high nest-box occupation (higher than 95%) and fairly good breeding success yearly (more than 70% of the eggs produced fledglings during first clutches). However, the capture and blood extraction caused some interference in the laying behaviour of both control and experimental females: 35.7% of females stopped current laying and begun a second clutch in the same nest-box, 13% did so in a different nest-box, and 15.6% abandoned the nest-box in the year of manipulation. We recorded the same information in years in which breeding females were not captured during laying (2001 and 2002) as an attempt to measure the extent of deleterious effects caused by the capture. The results in these not-manipulated females indicated that 14.3% of females stopped laying and begun a second clutch in the same box, 12.7% did so in a different nest-box, and 15.9% of females abandoned the nesting attempts. Therefore, the main disturbance effect of our experiment was an increase of the percentage of females that interrupted the laying sequence and begun a second breeding attempt in the same box (14.3% vs. 35.7%, respectively).

The manipulation of nests, ringing, bleeding and the greenery experiments were undertaken under licence from the Spanish Ministry of Environment and the Regional Government of Madrid.

Testosterone Assays

Females were captured in seven consecutive days of March in each year during laying (second or third

egg). We never spent more than 30 min between the placement of traps into the nest-boxes and the release of the females. Blood samples were taken within 10 min after capture, so females spent less than 20 min in the nest-box before handling. Time of handling and time in the nest-boxes before handling were similar in control and experimental females. Although a recent study has shown that T may increase rapidly in response to territorial intrusions (McGlothlin et al. 2008), our sampling protocol alternated captures between groups, and thus we expect that eventual differences in capture and handling timing were randomly distributed between groups. Females were captured from 7:30 to 12:30 h GMT, and there were not differences between control and experimental females in the time of capture.

We took blood samples of 800 μl from the jugular vein and placed them in heparinised tubes. 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. Testosterone was extracted with a volume of 10× diethyl ether from 200 μl plasma samples and resuspended in an equal volume of assay buffer [extraction recovery = 89% (SE = 3.4)]. For the assays we used a commercially available testosterone kit (ELISA, Cayman Chemical, MI, USA). Parallelism was tested in plasma pools and found satisfactory against the measured range. The bounds of detection (80–20% binding) in the standard curved were typically 12–400 pg/ml. Samples lower than the minimum detectable level (12 pg/ml) were assigned that minimum value. Samples with concentrations higher than 400 pg/ml were diluted in assay buffer and assayed in a second run. The within-assay coefficient of variation was 10.21%. A set of identical internal standards were run in each assay. The inter-assay variation, after correction by means of linear regressions of these standards, was 7.31%. The antibody used in the kit is highly specific for testosterone, but has some cross-reactivity with other androgens (5α-dihydrotestosterone: 27.4%; androstenedione: 3.7%).

Nesting Interferences

Direct measurements of female aggressiveness are very difficult to obtain in the wild. Therefore, a correlate of female competition (i.e. nesting interferences, see below) has been used in this study. The rationale of this analysis is the occurrence of nesting interference in nest-boxes. We collected data of nesting interferences by means of daily visits to control and experimental nest-boxes during laying, incubation and the first 2 d of the nestling period. In average we made 20 visits to each control and experimental nest-boxes (range 16–24 visits per box). Control and experimental nests were observed for similar periods of time in each of the three study years.

The nesting interferences considered in the study were included in three categories: (1) partial loss of material or eggs (i.e. loss or disarrangement of the nest building material, loss of single eggs during laying and/or incubation periods); (2) total breeding failure (i.e. loss of the total number of eggs and/or chicks younger than 2-d old); and (3) female interferences (i.e. female nest abandonment, changes in nest ownership by females, or fights between females in the nest-boxes). During each year, we used a categorical variable to record the occurrence of interferences in each nest-box. Nest-boxes suffering at least one type of interference were included in the positive group. The remaining nests were included in the negative group. Interferences caused by the capture of females (i.e. female nest abandonment or interruption of laying immediately after the capture) were not considered in the analysis. In cases of complete nest failure caused by the capture of the females, we simulated the natural behaviour of males by adding green plants to the experimental nests. Thus we could obtain data of 'natural' nesting interferences in all nest-boxes. In total, 162 events of interference occurrence (yes/no) were recorded (i.e. 27 nests × 2 treatments × 3 yrs).

We are confident that the great majority of cases of interference were due to female–female competition, as predation is rare at this time in breeding and we often found dropped eggs in the floor. During the laying period, neighbouring females visit each others' nests very frequently, and these visits often lead to fights, nest material pilfering and eggs sabotages. However, some nesting interferences could be the consequence of predation, thus increasing type II error in the interpretation of our results.

Statistics

The distribution of testosterone concentration followed a right-skewed distribution in both groups of females. Firstly, we compared each year the variances and coefficient of variation in T levels between control and experimental groups. Then, to achieve normality of the residuals of the ANCOVA models (see below), all testosterone values were Box-Cox transformed $(x_i + 0.04)^{0.05}$ before the analyses.

We used ANCOVA models to analyse the effect of the manipulation on testosterone levels in female starlings. Reproductive experience of females (inexperienced vs. experienced) was used as a categorical factor. We controlled for the possible effect of year, including this variable as a fixed factor. Laying date (calculated as number of days elapsed since the first egg appeared in the colony and/or year) and body mass were used as covariates in the analyses. Some females ($n = 18$) were recorded in more than one year in the colony. Thus, to avoid pseudo-replication, we randomly selected one single data point per female in the analyses.

We tested for differences in probability of occurrence of episodes of nesting interference (see above) by means of a combined test of probability (chi-square test) based on presence/absence data for each of the nests in the colony. Finally, we tested whether the presence of nest interferences was associated with female testosterone levels by using ANCOVA. We used year as a fixed factor in this analysis.

Results

Working with the original not-transformed data we observed that the addition of green material in the nest increased the variance and the coefficient of variation in testosterone levels between females in the experimental group with respect to the control group in all years (variances: 0.062 vs. 0.43 ng^2/ml^2, CVs: 5.81 vs. 8.37%, control vs. experimental; tests comparing variances: year 2004: $F_{21,23} = 70.5$, $p < 0.001$; year 2005: $F_{12,14} = 8.88$, $p < 0.001$; year 2006: $F_{17,14} = 4.37$, $p = 0.0039$).

The ANCOVA model explained a significant percentage of the variance in circulating testosterone levels of spotless starling females ($F_{13,65} = 2.23$, $R^2 = 38.68\%$, $p = 0.017$). There were no differences in the effect of the manipulation between experienced and inexperienced females ($F_{1,65} = 0.058$, $p = 0.81$). Experimental females had higher T levels than control females (means: 0.36 ± 0.04 vs. 0.68 ± 0.11 ng/ml, control vs. experimental: $F_{1,65} = 4.93$, $R^2 = 7.58\%$, $p = 0.029$). This effect was different between years (year × treatment: $F_{2,65} = 4.67$, $R^2 = 14.36\%$, $p = 0.013$; see Fig. 1). *Post hoc* tests showed that the manipulation induced an increase in circulating testosterone levels of experimental females during all years, although the significance changed between years: significant in 2005 ($F_{1,14} = 7.35$, $p = 0.017$), marginally significant in 2006 ($F_{1,17} = 3.36$, $p = 0.069$), whereas no detectable effect was seen in 2004 ($F_{1,32} = 0.51$, $p = 0.48$).

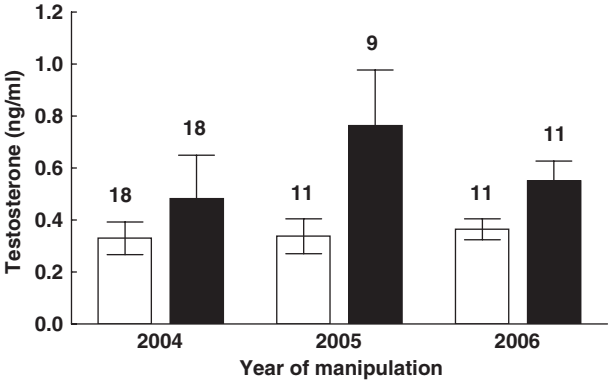

Fig. 1: Testosterone levels (mean ± SE and sample sizes) of control (white bars) and experimental (black bars) females by year of study. Original testosterone concentrations are presented in the figure, but Box-Cox transformed data were used in the analyses (see Section 'Methods').

Laying date, but not body weight, had a significant negative effect on testosterone levels (laying date: $F_{1,65} = 4.59$, $R^2 = 7.05\%$, $\beta = -0.27$, $p = 0.036$; body weight: $F_{1,65} = 1.43$, $R^2 = 2.20\%$, $p = 0.24$). The decrease in circulating testosterone with laying date was not different in control and experimental females (parallelism treatment × laying date: $F_{2,63} = 0.19$, $p = 0.83$; see Fig. 2).

The experimental addition of green plants caused a significant increase of nest interference in the three categories considered (partial loss of material or eggs: 21 vs. 35 nests, $\chi^2 = 5.35$, df = 1, $p = 0.021$; total breeding failure: 16 vs. 28 nests, $\chi^2 = 4.47$, df = 1, $p = 0.035$; female interferences: 12 vs. 33 nests, $\chi^2 = 13.57$, df = 1, $p < 0.001$). In addition, females who occupied nest-boxes with some nesting interference had higher level of testosterone that

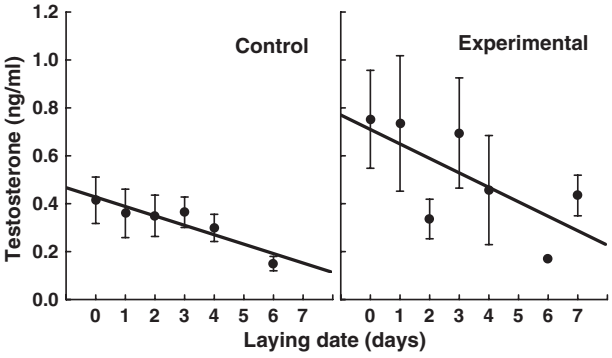

Fig. 2: Testosterone levels (means ± SE) of control and experimental females in relation to laying date. Original testosterone concentrations are presented in the figure, but transformed data in the analyses (see Section 'Methods').

females owner of nest-boxes without interferences (0.61 ± 0.10 vs. 0.35 ± 0.05 ng/ml: $F_{1,75} = 4.87$, p = 0.030). This effect was not different in the 3 yrs of the study (interaction interference × year: $F_{2,75} = 0.11$, p = 0.89; see Fig. 3) nor between control and experimental females (interaction interference × treatment: $F_{1,75} = 1.52$, p = 0.22).

Discussion

The experimental addition of green plants in spotless starling nests was successful in affecting female testosterone levels. Experimental females had higher values in both the mean and the variance of their circulating testosterone levels with respect to controls at the time of laying. There are two non-excluding explanations of these results. First, females might be responding to the manipulation itself (i.e. the green enhancement of nests), as nest greenery is a correlate of male quality (Brouwer & Komdeur 2004; Veiga et al. 2006). Second, females could increase T levels in response to the increase in female–female competition that might be brought about by the increase of green material (the courtship hypothesis; see Brouwer & Komdeur 2004). Both possibilities respond to the same ultimate mechanism of an increase in T levels in agreement with the courtship hypothesis. However, the link that we found between high testosterone levels and nest-box interferences would support the hypothesis that testosterone increases mainly as a result of the perceived female–female interactions (proximate mechanism) and less in preparation for an expected high competition between females in greenery nests (ultimate mechanism).

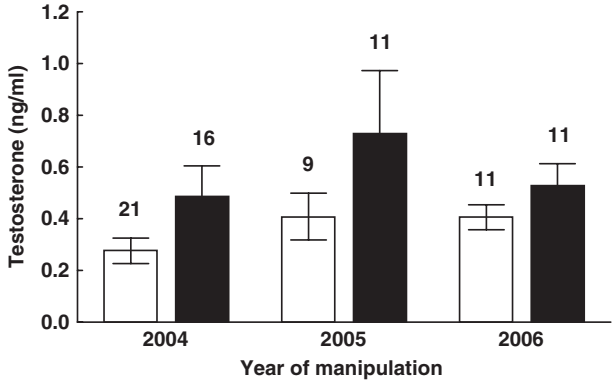

Fig. 3: Testosterone levels (means ± SE and sample sizes) of females by presence (black bars) or absence (white bars) of nesting interference in their nests-boxes and year of study. Original testosterone concentrations are presented in the figure, but transformed data in the analyses (see Section 'Methods').

An alternative explanation would be that T levels could be increased by direct exposure to the odour of the plants. Some recent results are intriguing in this sense, suggesting that the role of olfactory information on avian behaviour, physiology and reproduction is underestimated (e.g. Balthazart & Taziaux 2009).

Our results lead to us to speculate about a possible role of female testosterone levels in sex determination. In two different previous experimental studies conducted in the same species (Polo et al. 2004; Veiga et al. 2004), we reported, respectively, a male-biased primary sex ratio caused by the experimental addition of green plants in the nests, and a male-biased broods as a consequence of female testosterone implants (Veiga et al. 2004; see also Rutkowska & Cichon 2006). This coincidence of effects may suggest a functional link between testosterone levels and male-biased broods. Indeed, previous studies have hypothesised a role for maternal testosterone in the process of sex determination, although it is not yet clear how this mechanism may operate (Krackow 1995; Pike & Petrie 2003). Alternatively, testosterone levels need not be functionally related to sex determination, and the correlation between male-biased broods and high testosterone might be due to confounding factors. For instance, if green material is a sign of male quality, then females may increase testosterone levels to: (1) be able to respond to the increased female competition produced by an increase in male quality; (2) manipulate offspring phenotype by means of a hormonal maternal effect, as expected by the differential allocation hypothesis (Burley 1986; Gil et al. 2004; Marshall et al. 2005).

Plasma testosterone levels were nearly twice higher in experimental than in control females (40% higher when the medians of the distributions were compared), although the magnitude and significance of the effect of the manipulation was not equal in the three years of the study (45% higher in 2004, 60% higher in 2006 and 200% higher in 2005, see Fig. 1). As a similar effect was found when comparing the difference between nests with and without interferences, we conclude that our manipulation increased female–female competition, without inducing detrimental effects or leading to extreme responses in reproductive behaviour (Searcy 1988; Moreno et al. 1999; Veiga et al. 2002; Sandell 2007). However, the observed increase in nesting interferences caused by the manipulation is an indirect measure of the increase in female–female competition. Thus this interesting result deserves further exploration. In addition to the effect in mean levels,

we recorded an increase of variance in plasma testosterone level in experimental females in all study years. This high variance suggests that there are differences in plasticity between females to respond to the manipulation.

An association between elevated testosterone levels and female aggression has been observed in several other species (e.g. Wingfield et al. 1982; Schwabl 1992; Cristol & Johnsen 1994; Eens & Pinxten 2000). Moreover, experimental work in dunnocks (*Prunella modularis*; Langmore et al. 2002) has shown that polygynous females were more likely to increase their testosterone levels in response to aggressive interactions towards potential rivals. In the spotless starling, male parental investment by males mated to more than one female varies in relation to female breeding status (for instance Moreno et al. 2002 show higher levels of male parental care to primary females), so it would pay females to increase their testosterone levels in order to maintain a preferential position within the harem.

Testosterone levels were found to decrease with date of laying in both experimental groups (i.e. during laying and incubation periods). Because laying date is an important determinant of breeding success, generally constrained by environmental circumstances and by female phenotypic condition (Pietiainen & Kolunen 1993; Wendeln 1997; Bety et al. 2003; Drent et al. 2003), our results could fit with a functional relationship between circulating testosterone levels and female condition. Thus, early egg-laying females would be those with a higher ability in female–female competition, via the increase in circulating testosterone. Alternatively, as testosterone is related to intra-specific competition for nests, this decrease with season may be a consequence of the similar seasonal decrease in competition for nesting sites with date (Wiebe 2003).

To conclude, manipulating the amount of green material carried by the male in the spotless starling caused variations in female testosterone circulating levels. As the same manipulation had previously been shown to influence clutch sex ratio adjustment, we propose that there is a functional link between high testosterone levels, male-biased sex ratios and female resource-holding potential in intra-sexual competition.

Acknowledgements

The present work was supported by projects CGL2004-00126/BOS, CGL2005-05611-C02-01/BOS and CGL2008-02843/BOS (MCEI). The fieldwork was carried out under licence of the 'Consejería de Medio Ambiente y Desarrollo de la Comunidad de Madrid' and the 'Ayuntamiento de Manzanares el Real de Madrid'.

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