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Elevated testosterone levels affect female breeding success and yolk androgen deposition in a passerine bird

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ABSTRACT

Although it is well documented that testosterone (T) is an important mediator in the regulation of behaviour in male vertebrates, its functional significance in females is less understood. Experimentally increased T in adult female birds has been found to have both advantageous and detrimental effects on behaviour and fitness. In addition, T may also mediate maternal effects when it is deposited into the egg yolk, and variations in androgen concentration between eggs contribute to differences in offspring phenotype and fitness. In this study we examined the effects of experimentally elevated female T on reproductive success and yolk androgen deposition in the spotless starling. The administration of exogenous T in female spotless starlings before egg laying caused negative effects on reproductive performance: when compared to control females T-females laid fewer eggs and raised fewer chicks. We also found an effect of elevated female T on yolk androgen deposition: T-females laid eggs with greater amounts of yolk T than control females, whereas yolk androstenedione levels were not affected. Although some of these effects likely involved a direct interference of female T with female reproductive function, some of them could be due to effects operating in eggs through maladaptive high T levels.

1. Introduction

It is well documented that testosterone (T) is an important mediator in the regulation of behaviour in male vertebrates (Bouissou, 1983; Adkins-Regan, 1987). Particularly in birds, the effects of T on morphology, behaviour and physiology have been extensively studied and it has been shown that either T or the traits whose expression depend on this hormone have a function on male reproduction (Wingfield et al., 1990; Ligon et al., 1990; Wingfield and Hahn, 1994; Saino et al., 1995; Saino and Møller, 1995a,b; Zuk et al., 1995; Hasselquist et al., 1999; Ketterson and Nolan, 1999; Kimball and Ligon, 1999; Peters et al., 2002; Casto et al., 2001; Stoehr and Hill, 2001; Strasser and Schwabl, 2004; Day et al., 2006). Although T presence and action are clearly not exclusive to males, its functional significance in females is less understood (reviewed in Staub and Dee Beer, 1997). Recently there has been increasing research on female T circulating levels (Elekonich and Wingfield, 2000; Hau et al., 2000; Langmore et al., 2002; Clotfelter et al., 2004; Groothuis and von Engelhardt, 2005; Jawor et al., 2006, 2007), probably encouraged by the evidence that, as in males, female–female aggression is a frequent property of avian mating systems (Jawor et al., 2006; Kempenaers, 1994; Smith and Sandell, 2005; Veiga, 2002), and by the discovery that females influence embryo development through differences in yolk androgen deposition (Schwabl, 1993).

Experimentally increased T in females can lead to both advantageous and detrimental effects on behaviour and fitness. For example, T supplemented females have been shown to increase singing rates or repertoires (Nespor et al., 1996; Adkins-Regan, 1999), courtship behaviour (Lank et al., 1999), female–female aggression (Searcy, 1988; Adkins-Regan, 1999; Sandell, 2007), the ability to acquire and maintain a nesting site (Veiga and Polo, 2008), enhance their social status (Veiga et al., 2004; Sandell, 2007) and induce male-biased sex ratios (Veiga et al., 2004). In contrast with these positive effects, other studies provide evidence for detrimental consequences of increased T in females such as decreased male choosiness and female attractiveness (McGlothlin et al., 2004; Ketterson et al., 2005), delay on the onset of laying, reduction in clutch size and/or delay on molt (Searcy, 1988; DeRidder et al., 2002; Clotfelter et al., 2004; Veiga et al., 2004; Rutkowska et al., 2005; Veiga and Polo, 2008), reduction of nestling feeding rates (Veiga and Polo, 2008) and negative effects on the immune system (Duffy et al., 2000; Eens et al., 2000; Peters et al., 2002; Casto et al., 2001; Mougeot et al., 2004; but see Saino et al., 1995; Hasselquist et al., 1999; Greenman et al., 2005).

In addition to its potential effects in adult females, T may also mediate maternal effects when it is transferred to the egg yolk (Schwabl, 1993). Although there is experimental evidence that high levels of yolk T increase chick growth, begging behaviour and dominance in several species (Eising et al., 2001; Eising and Groothuis,

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2003; Lipar and Ketterson, 2000; Pilz and Smith, 2004), elevated yolk T was also found to delay hatching and reduce egg hatchability in some studies (Navara et al., 2005; Sockman and Schwabl, 2000), and negative effects or no effects of increased yolk androgens on phenotype or viability have also been reported (Andersson et al., 2004; Rubolini et al., 2006a,b; Sockman and Schwabl, 2000; Uller et al., 2005). Furthermore, several studies have shown that androgens may have negative effects on the immune system of both adults and nestlings (and juveniles) and may increase the incidence of parasites (Duffy and Ball, 2002; Eens et al., 2000; Müller et al., 2005; Uller and Olson, 2003), suggesting that these constraints may limit the amount of hormones that females may allocate to eggs or that offspring may tolerate (Gil, 2003, 2008). In agreement with these costs, evidence has found that yolk androgen deposition varies in relationship to female quality and condition (Gil et al., 2004a; Gil et al., 2006; Schwabl, 1993; Pilz et al., 2003). The fact that variations in androgen concentration between eggs contribute to differences in offspring fitness suggests that an accurate control of this transfer should have evolved in the absence of physiological constraints (Lipar et al., 1999). However, it is not known yet how exactly androgens enter the egg and whether this transfer is purely a passive process based on diffusion into the yolk's lipophilic matrix or an active mechanism controlled by laying females (Sockman et al., 2006). Although some correlative studies have shown a positive relationship between female plasma and yolk T levels (Schwabl, 1996; Whittingham and Schwabl, 2002) other studies provide negative evidence for such relationship (Mazuc et al., 2003; Navara et al., 2006; Verboven et al., 2003). To our knowledge only two studies have addressed this question by experimentally increasing female plasma T (Clotfelter et al., 2004; Rutkowska et al., 2005) and evidence so far is not uniform: although in both studies, yolk T increased in eggs from T-treated females, injections of T in laying zebra finches increased yolk steroid to a very limited extent (only an 0.025% of the injected dose) suggesting that transfer of hormones from plasma to yolk is unlikely to play a large role in the determination of yolk androgen levels (Rutkowska et al., 2005). In contrast, eggs laid by T-implanted dark-eyed junco females showed significantly more yolk T than eggs laid from control females (Clotfelter et al., 2004).

In the present study we manipulated T levels in female spotless starling in order to examine the relationship between female T levels and yolk androgen deposition and to determine the effects of increased female T on reproductive performance. We used subcutaneous implants to elevate plasma T levels in free-living females and monitored changes in the following reproductive traits: laying date, clutch size, egg size, yolk androgen content, number of hatchings and number of fledglings. Since in the spotless starling the addition of exogenous testosterone has been suggested to reduced female fitness (Veiga and Polo, 2008) we expected testosterone-females to present lower reproductive performance than control females.

2. Methods

2.1. Field procedures

The study was conducted in a nestbox colony of spotless starling in Soto del Real, near Madrid, in central Spain, from March to July 2006. The spotless starling is a facultatively polygynous passerine closely related to the European Starling (*Sturnus vulgaris*). It is commonly double brooded and females lay the first clutch around mid-April and the second clutch around the end of May. The floater population is numerous and intra-sexual competition for nesting sites is strong in males and females so both sexes defend nest boxes against competitors. Incubation usually starts before clutch completion. The main part of chick feeding is performed by the female (Veiga, 2002).

Beginning approximately a month before egg laying starts, females were trapped when visiting the nest boxes and randomly assigned to either an experimental (T-females) or a control group (C-females) ensuring the same distribution of capture dates among treatments (capture dates ranges: C-females from March 28 to April 19th; T-females from March 27 to April 17th; $F_{1,34} = 0.017$, $p = 0.90$). T-females received a subcutaneous implant of a piece of 10 mm long Silastic tube (1.95 mm outer diameter, 1.47 mm inner diameter; Dow Corning) filled with T propionate (Sigma; implanted T on average weighed 0.0122 ± 0.002 g), while C-females received an empty implant. Implants were sealed at both ends with medical silicone, and were implanted on the right side of the back between the wings while the birds were under local anaesthesia. The dose of testosterone we chose to administer was based on the finding that in some bird species, including the European starling (a sister species of the spotless starling), levels of circulating plasma testosterone are about four or five times lower in females than in males during the spring maximum (Silverin and Wingfield, 1982; Sandell, 2007). Thus, we used a fifth of the dose that we had used before to induce changes in territorial behaviour and parental investment in males of the same species with no adverse effect (Moreno et al., 1999). This dose has indeed been successfully used in females of the same species (Veiga et al., 2004; Garcia-Vigón et al. (2008)) and has shown not to interrupt reproductive activities. In a preliminary study in our population, the testosterone plasma concentration of females treated with this dose of testosterone one month after implantation (mean ± SD: 28.5 ± 11.43 days) was 1.2–0.55 ng/ml (range 0.13–2.79 ng/ml; $n = 9$) for T-females and 0.36 ng/ml (range 0.04–1.08 ng/ml; $n = 53$; López-Rull and Gil, unpublished data) for C-females. The mean level recorded in T-females was similar to the maximum levels measured in non-manipulated females. Thus, the implants probably led to a relatively high level of testosterone in the circulating plasma of females. The experimental protocol was approved by the Environment Department of the Autonomous Community of Madrid and was done in compliance with the European Communities (Council Directive of 24 November 1986 (86/609/EEC).

Capture and manipulations of females were discontinued approximately a week before the beginning of egg laying in the population. In total we implanted 20 T-females and 17 C-females. Because a second capture in a sensible stage of their breeding cycle such as pre-laying (one week before laying) and laying causes undesirable perturbations on laying or nest abandonment (Veiga et al., 2004), we did not attempt to capture females and thus a blood sample after the implant was not collected. However, in a limited subsample of recaptures during chick feeding (mean 56.67 ± 23.10 days after manipulation) implants of 6 experimental females were found to contain no traces of the hormone, indicating that they had liberated their content into the circulating plasma. A blood sample was taken in this subsample of females (6 T-females and 4 C-females) in order to determine the consequences of the manipulation in circulating T levels.

When captures and manipulations were discontinued, observations were performed in order to assess the identity of females breeding at each nest box. Out of 20 implanted T-females and 9 out of 17 C-females breed in our colony. Since the proportion of breeding females was similar between groups (55% and 53% respectively; Chi-square = 0.04, $p = 0.834$, df = 1) we assume that our manipulation affected both groups equally. When laying approached, nest boxes were visited daily to determine the date at which the first egg was laid and the final clutch size. Eggs were marked with a non-toxic marker as they were laid and measurements of egg length and width were taken with a digital calliper to the nearest 0.01 mm.

Before incubation started, approximately 25 mg of yolk was taken from the first egg laid in clutches belonging to manipulated females. Although yolk androgens in our study population have

been shown to vary in relation to laying order (Lopez-Rull and Gil, 2009) differences in androgen content have also been found to be greater between than within clutches, thus allowing us to estimate mean clutch concentration from a single egg biopsy (Lopez-Rull et al., 2008; Lopez-Rull and Gil, 2009). Biopsies were done by inserting the 25-gauge needle of a winged infusion set into the yolk, while the egg was being candled. After sample removal the hole was sealed with a tiny strip of flexible wound dressing (Opsite, SmithandNephew, Hull, UK) so the embryo could continue its normal development. Yolk samples were individually weighed in the lab, homogenized in 1 ml of distilled water and frozen at −70 °C for future androgen analysis. All nests were monitored periodically and nestlings were ringed, measured and weighed at 14 days of age.

2.2. Hormone analyses

We examined data for two yolk androgens, A4 and T which we consider to be the most biologically relevant; A4 because it is the most concentrated androgen in starling eggs and T because previous research has shown that yolk T enhances bird chick growth and begging (Schwabl, 1996; Eising et al., 2001). Yolk steroids were extracted by adding 3 ml of a mixture of petroleum and diethyl ether (40:60) to the sample, vortexing for 15 min and centrifuging for 10 min (4 °C, 2000 × 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. The dried extract was redissolved in 1 ml of ethanol 90% and kept at −20 °C overnight and centrifuged for 10 min (4 °C, 2000 × RPM) to remove further proteins. Supernatants were dried under a stream of nitrogen, and the dried extract was redissolved in steroid free serum (DRG Labs, Germany). Calculated recoveries in cold spiked samples showed high recoveries (>90%), and thus values were not corrected by individual recovery estimates.

Yolk concentrations of testosterone (T) and androstenedione (A4) were determined by two different EIA kits highly specific for each hormone (DSL Labs, USA for T and DRG Labs, Germany 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 12.80% and for A4 the intra-assay coefficient of variation was 3.30%.

Steroid extraction from 300 μl plasma samples was performed by a single diethyl ether extraction (3 ml), involving vortexing, freezing, decanting, drying and re-suspension in a 300 μl volume of assay buffer (Cayman Chem., USA). Samples were assayed using a highly sensitive T assay (Cayman Chem., USA). Intra-assay coefficient of variation was 7.33% as measured from duplicates.

2.3. Data analyses

In order to rule out the possibility that differences in reproductive success and yolk androgen deposition were due to differences in female quality between groups, we used *t*-tests to analyze differences in female quality prior to the manipulation. Female age was estimated by measuring female throat feather length, which is an age dependent trait, related to reproductive success in this species (Lopez-Rull et al., 2007). Differences in female body mass prior to the manipulation 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. Differences between treatments in female T levels, reproductive performance, and yolk hormones were analyzed using one-way ANOVAs. Laying date was measured as the number of days elapsed after the first breeding attempt recorded in the colony during the current year. Egg volume was calculated by the formula: $0.45 \times \text{length} \times \text{width}^2$ (Worth, 1940). Hatching success was estimated as number of hatchings/clutch size. Fledging success was estimated as number of fledglings/number of hatchings, and breeding success was estimated as number of fledglings/clutch size. Statistical analyses were performed with SPSS v.11.5.

3. Results

3.1. Hormone implants and female attributes

Prior to manipulation, females assigned to either experimental group did not differ in throat feather length ($F_{1,34} = 0.40$, $p = 0.53$) or body mass ($F_{1,31} = 0.84$, $p = 0.77$), indicating that female attributes were randomly distributed between T-females and C-females. No differences were found in laying dates between T-females and C-females ($F_{1,17} = 1.32$, $p = 0.26$; power of the test = 0.20; Fig. 1).

The experimental treatment resulted in differences in plasma androgen concentrations between groups. In a subsample of females captured during chick feeding, T-females had higher testosterone concentration than C-females (T-females 1.52 ± 1.11 ng/ml compared to C-females 0.11 ± 0.02 ng/ml, $F_{1,8} = 9.61$, $p = 0.01$; Fig. 1). Plasma *T* values of a limited number of implanted females captured in the following breeding season showed that differences between groups were no longer present (T-females 0.3 ± 0.02 ng/ml compared to C-females 0.5 ± 0.19 ng/ml, $F_{1,10} = 0.641$, $p = 0.434$; Fig. 2).

3.2. Reproductive success

T-females laid smaller clutches than C-females (Table 1). Egg volume tended to be greater in T than in C-females but differences were not significant (Table 1). T-females also exhibited reduced hatching success (number of hatchings/clutch size) and diminished breeding success measured as number of fledglings/clutch size when compared to C-females (Table 1). Fledgling success measured as number of fledglings/number of hatchings was not different between groups (Table 1).

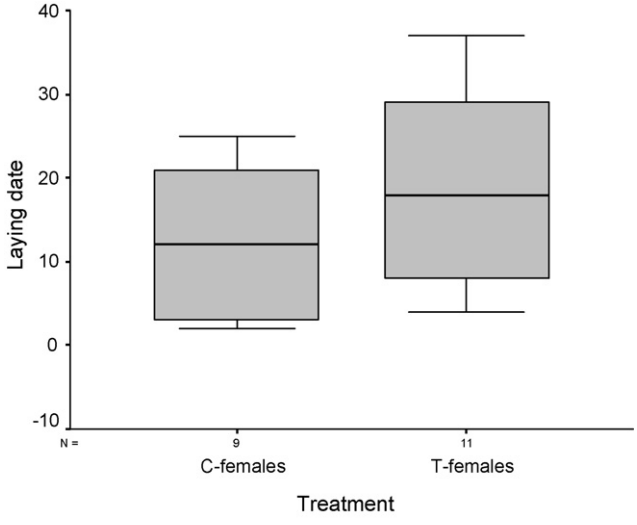

Fig. 1. Box plot diagram for laying dates in C-females and T-females.

Table 1
Reproductive success of T-females ($n = 11$) and C-females ($n = 9$).

		Mean	SD	F	p
Clutch size	C-females	5.33	1.00		
	T-females	3.90	1.30	7.25	**0.015**
Egg volume (cm^3)	C-females	5.64	0.33		
	T-females	5.95	0.25	3.52	0.085
Hatching success	C-females	0.76	0.31		
	T-females	0.42	0.36	4.87	**0.040**
Breeding success (fledglings/clutch size)	C-females	0.66	0.38		
	T-females	0.26	0.35	5.96	**0.025**
Fledgling success (fledglings/hatchings)	C-females	0.84	0.35		
	T-females	0.62	0.52	0.98	**0.34**

Hatching success is measured as the number of chicks hatched/clutch size. Breeding success is measured as the number of fledglings/clutch size. Fledging success is measured as the number of fledglings/hatchings.

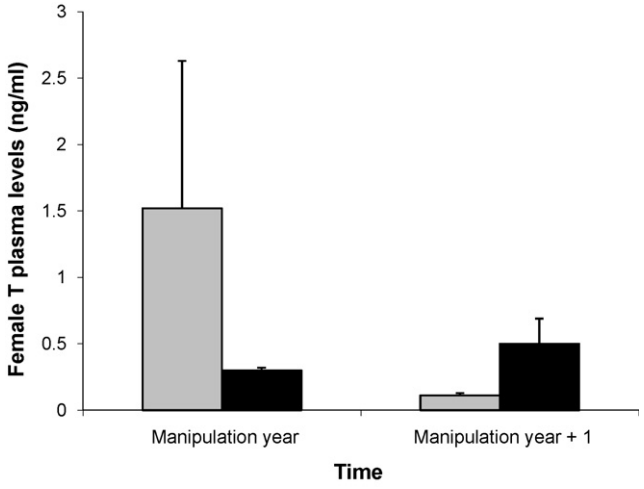

Fig. 2. Mean plasma T concentration (±SE) in T-implanted females (grey bars) and empty-implanted females (black bars) during the manipulation year (MY) and one year after (MY + 1).

3.3. Yolk androgen deposition

We found an effect of the treatment on yolk androgen deposition: T-females deposited higher levels of testosterone in their eggs than C-females (T-females 5.60 ± 1.73 ng/ml compared to C-females 3.30 ± 0.95 ng/ml, $F_{1,9} = 8.41$, $p = 0.01$; Fig. 3). Yolk A4 did not differ between treatments (T-females 8.21 ± 3.87 ng/ml compared to C-females 7.83 ± 3.07 ng/ml, $F_{1,11} = 0.043$, $p = 0.84$).

4. Discussion

Our experiment showed an effect of elevated female T on the deposition of androgens in the eggs: T-females laid eggs with greater amounts of yolk T-females than C-females but no differences were found in yolk A4. Also, the administration of exogenous T in female spotless starlings before egg laying reduced reproductive success: when compared to C-females, T-females laid fewer eggs and raised fewer chicks.

In contrast to a recent study conducted in this species (Veiga et al., 2004) we found no differences in the onset of laying between groups, confirming that differences between groups in reproductive success were induced by the experimental manipulation and were not consequences of delayed breeding. Some of these negative effects, such as reduced clutch sizes, are likely due to an interference of high androgen levels with physiological reproductive function in females (Rutkowska et al., 2005), however, we cannot fully distinguish whether decreased nestling survival is due to effects operating in females or in eggs. For example, the reduction in hatching success in T-females could be a consequence of suboptimal incubation (likely induced by the administration of exogenous T) which cannot be separated from egg composition effects, since eggs were not cross-fostered in our study.

Detrimental effects on reproductive performance of implanted T-females have been reported in several species. For example, experimental high plasma T levels have been shown to decrease female attractiveness (Ketterson et al., 2005), delay the onset of laying, reduce clutch size (Clotfelter et al., 2004; Rutkowska et al., 2005; Searcy, 1988), delay moult (Clotfelter et al., 2004; DeRidder et al., 2002) and even depress the immune system (Duffy and Ball, 2002). On the other hand, studies examining the effects of increased androgen content in egg yolks have found contrasting results for nestling fitness, positive in some cases but negative in others. Negative consequences of increased yolk androgen concentration include decreased growth, reduced survival and impaired immunocompetence (Andersson et al., 2004; Müller et al., 2005; Navara et al., 2005; Rubolini et al., 2006a,b; Sockman and Schwabl, 2000; Uller et al., 2005; Uller and Olson, 2003). In contrast, a previous study in the same species and population as the present study which involved T injections into eggs reported a net growth benefit in nestlings (Müller et al., 2007), in line with other studies in other species (Eising et al., 2001; Eising and Groothuis, 2003; Lipar and Ketterson, 2000; Pilz and Smith, 2004). One possibility to explain the difference of outcomes between our results and those of the egg injection experiment lies in the difference in doses that were trans-

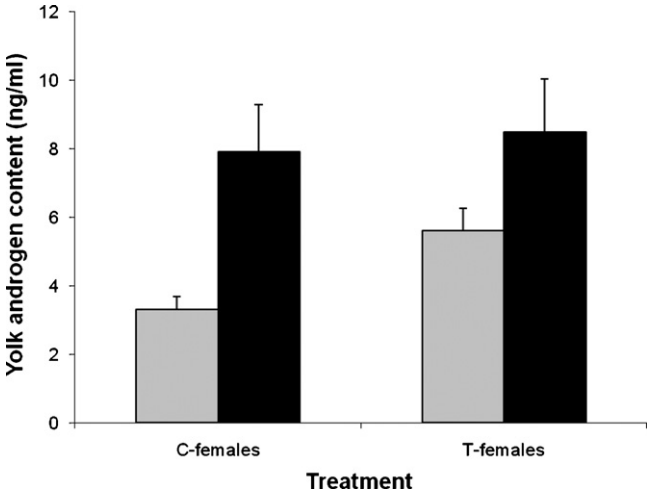

Fig. 3. Mean yolk androgen concentration (±SE) (T grey bars and A4 black bars) of T-females ($n = 5$) and C-females ($n = 6$).

ferred into the eggs. Whereas the egg injection involved an increase in androgen levels of 1 SD, our implants resulted in an increase of 3 SD, in other words almost twice that found in control eggs. A previous study has suggested that the effects of yolk androgens may likely involve an inverted-U shaped dose-dependency (Navara et al., 2005), and in that sense some of our results (decreased survival) could be explained by detrimentally high levels of yolk androgens. However, we cannot discard an additional effect of androgens on poor maternal performance.

Numerous studies have suggested a role of androgens in mediating aggressive behaviour in females (Eens et al., 2000; Petrie, 1983; Staub and Dee Beer, 1997; Wingfield et al., 1987; but see Schwabl et al., 1988; Elekonich and Wingfield, 2000; Hau et al., 2000; Jawor et al., 2006). Two experimental studies with song sparrows (*Melospiza melodia*) and bluebirds (*Sialia sialis*) (Elekonich and Wingfield, 2000; Navara et al., 2006) found that plasma T levels were significantly lower in females experiencing aggressive interactions than in control females, and similarly, in the red-winged blackbird (*Agelaius phoeniceus*) T decreased before the period of territorial and aggressive behaviour ends (Cristol and Johnsen, 1994). These studies showed that the decrease in T levels may be adaptive because high T levels may result in interruptions of behaviours that are necessary for a successful breeding attempt. In many species, there is evidence that experimentally elevated T tends to increase the occurrence of behaviours related to mating while decreasing self-maintenance and parental care. Several experimental studies have found that females showed increased aggression after T-implantation in red-winged blackbirds (Searcy, 1988), dark-eyed juncos, *Junco hyemalis* (Zysling et al., 2007) and European and spotless starlings (Sandell, 2007; Veiga et al., 2004). Because in the spotless starling, male feeding contribution is critical for future offspring survival (Moreno et al., 1999) and males allocate most of their parental effort to their primary females' brood, intrasexual competition between females is very high. A recent study in this species (Veiga and Polo, 2008) suggested that T conferred a significant advantage for females to get and maintain a nesting territory but interfered with reproductive investment and parental care causing a reduction of fitness. In accordance, it has been proposed that, if the maintenance of high levels of androgens is costly for females, then the transfer of excess T into eggs would be an effective method of regulating circulating T at basal levels and thus avoiding such detrimental effects (Navara et al., 2006). This mechanism would explain the fact that T-females allocated more T into their eggs than C-females despite the costs of elevated androgens to the offspring. However, it is unlikely that such a mechanism would have evolved for that function, since its usage would be exclusively restricted to a very short period of the female's life, namely that of oogenesis.

Environmental factors are known to modulate the production of steroid hormones in birds (Nelson, 1995), and female birds have been shown to adjust yolk androgen content in response to social and environmental changes (Gil et al., 1999, 2004b; Groothuis and Schwabl, 2002; Mazuc et al., 2003; Müller et al., 2002; Navara et al., 2006; Reed and Vleck, 2001; Schwabl, 1997; Tanvez et al., 2004; Verboven et al., 2003; Whittingham and Schwabl, 2002). These findings suggest the existence of a control of maternally derived yolk androgens. Hackl et al. (2003) found that after injecting radioactively labelled T to Japanese quail females (*Coturnix coturnix japonica*) a very limited amount of radioactivity could be found in yolks, indicating that steroids injected in the blood stream do not easily reach the egg. Based on this result Rutkowska et al. (2005) proposed that social cues may activate androgen production at hypothalamic levels, and that these levels in turn may modify androgen secretion in different glands. While the physiological mechanism behind yolk androgen deposition remains unclear, we should expect a trade off between the amount of T that females may produce/maintain and the amount of T that offspring may tolerate without incurring in detrimental effects.

In normal conditions, there is a positive relationship between the two main androgens in avian eggs (T and A4) in the starling. However, T-females laid eggs with almost double T concentration than controls, while no differences were detected in yolk A4. This suggests that the experimental implantation of T did not lead to a general up-regulation of androgen production in the follicles, but rather to a very specific T increase. Although previous studies using bolus injections of T suggest that direct transfer of T from plasma to egg is low Hackl et al., 2003; Rutkowska et al., 2005), it is possible that a continuous elevation of T levels as achieved by our implants could result in a higher transfer. Alternatively, yolk T production at the follicle level could have been boosted by the increased plasma levels. Clearly further work is required to understand the dynamics of yolk hormone deposition and its relationship with circulating hormone levels.

We did not measure circulating T levels in females during laying or shortly after and thus we cannot rule out the possibility that our manipulation did not raise T levels above the maximum values found under not manipulated conditions. However, because C-females and T-females were equally likely to breed we assume that our manipulation did not induce severe detrimental effects such as inhibition of reproduction, perturbations on laying or a higher propensity to abandon the nest (Moreno et al., 1999; Searcy, 1988; Veiga, 2002). An interesting result is the fact that when a subset of females was captured to check T implants and resultant testosterone levels, implants no longer contained crystalline steroid, but the circulating T levels were still elevated. This may indicate that our manipulation caused a long-lasting effect, which could be due to the use of T propionate, whose effects have a longer permanence. Interestingly, differences were not detectable a year later, which suggests that life-long effects of yolk T in females of this species (Veiga and Polo, 2008) are not due to a long-lasting increase of plasma T. Further research should examine whether these effects are due to increased sensitivity of androgen receptors, or rather to a permanence of social effects created in the first year of manipulation.

In agreement with previous studies, T-females laid fewer eggs than C-females and egg volume tended to be higher although differences were not significant (Clotfelter et al., 2004; Rutkowska et al., 2005; Searcy, 1988; Veiga et al., 2004). This tendency of increased egg volume could be explained by the existence of a physiological factor behind the trade off between egg size and clutch size or alternatively by the fact that, since high T levels inhibit ovulation, more resources would be deposited in fewer eggs (Rutkowska et al., 2005). Another plausible explanation is that the ability of T-females to lay bigger eggs is related to a differential provisioning of eggs with nutrients and/or androgens in relationship to sex allocation (Cordero et al., 2001; Müller et al., 2002; Rutstein et al., 2005). A previous study has shown that in spotless starlings T-females produced more sons than C-females (Veiga et al., 2004), so it is possible that such a mechanism could also be operating here.

In summary, female spotless starlings increased yolk testosterone deposition and reduced their reproductive success when faced with an experimental increase in plasma testosterone levels. Although some of these effects likely involved a direct interference of female T with female reproductive function, others could be due to effects operating in eggs through maladaptive high T levels. These results suggest that experimental increases in circulating T cannot be fully controlled by females, and result in detrimental effects in nestling growth. Further research should examine whether this lack of control also extends to natural variation in female T levels, or is it restricted to artificially high levels caused by implants.

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