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Testosterone, cuckoldry risk and extra-pair opportunities in the Seychelles warbler

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In male birds, testosterone (T) plays an important role in aggressive and mate-attraction behaviour. In the cooperatively breeding Seychelles warbler, *Acrocephalus sechellensis*, extra-group copulations (EGCs) occur frequently, but are not accompanied by sexual courtship displays as in within-pair copulations. Paternity is nearly always gained by primary males. We investigated whether T levels and sperm storage capability (cloacal protuberance (CP)) in adult primary and subordinate males were related to timing of egg laying, levels of cuckoldry and extra-group paternity (EGP) opportunities. During the sexually active period before egg laying, T levels and CP were only elevated or enlarged (respectively) in primary males, and some suggestion was found that subordinate males do not invest in elevated T levels. The peak in T occurred during the fertile period of the female partner and corresponded to the peak period of male sexual displays and mate guarding, but was independent of cuckoldry risk (density of neighbouring primary males). CP was also enhanced during this period; however, CP but not T remained elevated after egg laying by their mates, and CP but not T was positively related to EGP opportunities (density of neighbouring fertile females). We conclude that T is involved in sexual courtship displays and mate guarding, but not in gaining EGCs. These findings contrast with those in other species where EGP involves elaborate sexual displays.

Keywords: testosterone; sperm storage; Seychelles warbler; status; paternity; cooperative breeding

1. INTRODUCTION

In avian species, as in other vertebrate species, the steroid hormone testosterone (T) plays a key role in the regulation of male reproductive behaviour, i.e. stimulating aggressive and mate-attraction behaviours (Moore 1984; Ketterson & Nolan 1994, 1999) and inducing secondary sexual characteristics (Wingfield *et al.* 1990; Weatherhead *et al.* 1993; Kimball & Ligon 1999; Buchanon *et al.* 2001; Setchell & Dixson 2001; reviewed by Nelson 2000). In birds, various factors have been suggested to influence male T levels including male–male competition for resources such as breeding territories, female partner fertility, opportunities for extra-pair copulations (EPCs) and, within cooperative breeders, male status. Male birds are normally highly sensitive to the fertility status of their mate and show a rise in T levels just before their mates become fertile (Wingfield *et al.* 1990; Mays *et al.* 1991; Schoech *et al.* 1996*a*). This peak in T is suggested to regulate the male's reproductive behaviour (Wingfield & Moore 1987; Wingfield & Farner 1993). During the female's fertile period, paired males are also sensitive to the risk of cuckoldry, i.e. mate guarding increases with increased intrusion pressure from extra-pair males (e.g. Alatalo *et al.* 1987; Møller 1987; Gray 1996; Komdeur *et al.* 1999). The rise in T levels during the mate's fertile period may, therefore, be associated with the risk of cuckoldry, e.g. the density of neighbouring adult males. A high T level may induce behaviours which reduce the male's risk of being cuckolded (e.g. Wingfield & Moore 1987; Birkhead & Møller 1992; Schoech *et al.* 1996*b*; Ketterson & Nolan 1999; de Ridder *et al.* 2000), e.g. heightened territoriality (Watson & Parr 1981; Chandler *et al.* 1994; Wingfield 1994*a*,*b*; Hunt *et al.* 1997; Van Duyse *et al.* 2000), within-pair courtship displays (Beletsky & Orians 1987; Enstrom *et al.* 1997; Peters 2002) and mate guarding (Saino & Møller 1995).

Elevated T levels could also be responsible for inducing behaviour or physiological changes (i.e. increased sperm production) that result in increased extra-pair fertilizations (EPFs) (Chandler *et al.* 1994; Raouf *et al.* 1997; Van Duyse *et al.* 2000; de Ridder *et al.* 2000). In many avian species, males increase reproductive success by seeking EPCs, resulting in EPFs (e.g. Westneat *et al.* 1990; Griffith *et al.* 2002). Levels of T may be influenced by the opportunities for EPCs, i.e. the density of neighbouring fertile females. In the superb fairy-wren (*Malurus cyaneus*), a species with a high level of EPFs, EPCs are mediated through sexual courtship displays (Mulder 1997). In this species, a positive association between male T levels and the number of fertile females in the population has been

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observed (Peters *et al.* 2001). However, given the tight association between EPC and courtship displays in this species, it is unknown whether the observed increase in male T levels is necessary for the expression of courtship displays, EPC behaviour, or both. The study on the superb fairy-wren is the only one, to our knowledge, to have investigated the relationship between male T levels and EPC opportunities so far. In the present study on the Seychelles warbler, *Acrocephalus sechellensis*, we attempt to resolve whether male T level is associated with courtship displays, EPC behaviour, or both.

The Seychelles warbler is a passerine bird endemic to the Seychelles islands in the Indian Ocean. Like many other tropical bird species, the socially monogamous Seychelles warbler is long-lived (average life expectancy is 5.5 years; Komdeur 1992), inhabits year-round stable territories with the same mate (Komdeur 1992) and, consequently, might have low T levels year-round. Alternatively, given the Seychelles warbler's pattern of seasonal reproduction (Komdeur 1996), T levels may show a seasonal peak. Although warblers can breed independently in their first year, a lack of suitable independent breeding opportunities drives some individuals into becoming subordinates within their own territory (Komdeur *et al.* 1995). In groups consisting of adult primary and subordinate males, within-group paternity is nearly always gained by the primary male (Richardson *et al.* 2001). Furthermore, extra-group paternity (EGP) is high (40%) and is always gained by primary males (Richardson *et al.* 2001). To minimize paternity loss, primary males guard their females intensively during their fertile period and expel intruding males aggressively from the territory (Komdeur *et al.* 1999). In contrast to the superb fairy-wren, males gain EPCs by surreptitiously intruding into territories and do not perform overt courtship displays to extra-group females (Komdeur *et al.* 1999).

In this study, we investigate whether cuckoldry risk and EGP opportunity affect T levels and sperm storage capacity in primary and subordinate male warblers. We measure cloacal protuberance (CP), an anatomical structure that results from the enlargement of the seminal glomerus (the site of sperm storage; Lake 1981), which accurately reflects sperm storage capacity and copulation frequency (Nakamura 1990; Birkhead *et al.* 1991; Briskie 1992). First, we investigate whether subordinate males are hormonally sexually inactive. We do this by comparing T levels and sperm storage between primary and subordinate males. Given that primary males sire nearly all young in the population, we predict that primary males have significantly higher T levels and more developed sperm storage organs than subordinate males. Second, we study whether T level is associated with either extra-group copulations (EGCs) *per se*, or with sexual displays, or both. The Seychelles warbler provides an excellent opportunity to do this as in this species obtaining within-group paternity requires sexual displays, whereas obtaining EGP does not. Therefore we predict that primary male T levels are high during their mates' fertile period, but are independent of EGP opportunities. Furthermore, we predict that sperm storage capacity is high during the female mates' fertile period but is also positively associated with EGP opportunities.

2. MATERIAL AND METHODS

(a) *Study population*

The Cousin Island population of Seychelles warblers has been monitored intensively since December 1985. During this time, almost all birds were individually colour-ringed (using a unique combination of three ultraviolet-resistant colour rings and a British Trust for Ornithology metal ring) and monitored throughout breeding attempts. Therefore, the reproductive history, putative pedigree, age and status of most Seychelles warblers are known.

The present study was based on the 2002 breeding season (July–September). We focused on adult birds only; i.e. birds older than eight months of age (the minimum age at which birds can produce young; Komdeur 1997). Each territory was checked for nesting activity, at least once every two weeks, by following the resident female for 15 min (Komdeur 1992). The identity of all birds present in each territory was recorded. Active nests were monitored throughout the breeding cycle to ascertain the egg-laying date. The status of all adult birds was based upon field observations combined with the available long-term demographic data. The 'primary' male and female were defined as the dominant, pair-bonded male and female in the territory while the term 'subordinate' included all other adult birds resident in the territory (Richardson *et al.* 2002). The female's fertile period is taken as the period starting 6 days before egg-laying until the day of laying the penultimate egg (Arvidsson 1992). In the Seychelles warbler, where a one-egg clutch is normal, the fertile period is assumed to span the 6 days up to the laying of the egg (Komdeur *et al.* 1999).

We assessed the opportunities for EGP available to the males within each territory. Seychelles warbler females only copulate successfully with extra-group males during their fertile period (Komdeur *et al.* 1999). Most EGCs took place with females on adjacent territories (81.3%, $n = 16$; Komdeur *et al.* 1999) and most extra-group young were sired by males from nearby territories (within two territories distance: 61.9%, $n = 21$; Richardson *et al.* 2001). Although EGP can take place over longer distances, this does not occur very frequently; therefore to simplify our calculations we estimated the opportunities for EGP within 50 m radius (approximately two territories) of the male's territory. For each male, we counted the number of fertile females (primary and subordinate females combined) in territories within 50 m radius of the male's territory on the day the male was caught. In most cases, we found none or one fertile neighbouring female and in only one case were two present. Therefore, we used the presence or absence of any fertile neighbouring females as the independent variable in the analyses. We assessed cuckoldry risk as the number of adult primary males (as only primary males gain EGP) present in territories within a 50 m radius of the male's territory on the day the male was caught.

(b) *Bird capture: challenges and sampling*

Birds were caught using mist nets. During the first 15 min session of catching no challenge was used. To facilitate catching a bird when it was not caught during the first session, an audible challenge was used at the net during the second 15 min session. The challenge used was a continuous playback of a Seychelles warbler song. Sessions with no challenge followed by a series of challenges were repeated (on different days) until the bird was caught. A bird was defined as being 'not challenged' when it was caught in the mist net during the first 15 min session of catching without a challenge, and 'challenged' when the bird was caught in the mist net during the audible challenge or within the 15 min between consecutive challenges. Capture is defined as when the bird flew into the mist net, and extraction as when the bird was taken out of the mist net. Upon extraction, the following were noted: the number of challenges used before capture, if challenged, whether or not capture took place during or between challenge sessions, the time lapse (±1 min) between the start of the first challenge and capture (capture time), and the time-lapse (±10 s) between capture and blood sampling (handling time) were noted. Blood samples (*ca.* 100 μl) were collected by brachial venipuncture, placed in a 1.5 ml screw-cap microfuge tube containing 3 μl of heparin and then immediately centrifuged at 3000 r.p.m. for 7 min. The amount of plasma (supernatant) obtained was quantified (μl), diluted in 1 ml of 100% ethanol in a screw-cap microfuge tube and stored at room temperature. The remaining centrifuged red blood cells were diluted in 1 ml of 100% ethanol in a screw-cap microfuge tube and stored at room temperature. DNA extracted from the red blood cell samples (following Richardson *et al.* 2001) was used to confirm the sex of each individual using the molecular (polymerase chain reaction) sexing method devised by Griffiths *et al.* (1998).

For each male, the width (w) and height (h; from the anterior end) of the CP was measured using callipers (±0.1 mm). Because the CP resembles a barrel shape from its anterior to posterior end, the volume of the protuberance (mm^3) was calculated as $h^* \times r^2$, where r was estimated as the $w/2$ (Mulder & Cockburn 1993). Both the T level and CP were determined for 29 primary and 11 subordinate males; however, only the T level was determined for another five primary and two subordinate males, and only the CP for another three primary and three subordinate males. For these males, age, based on birth dates, was calculated in years.

(c) *Testosterone analyses*

The diluted plasma was centrifuged at 14 000 r.p.m. for 2 min and the supernatant was poured off and used for the assay. A competitive-binding radioimmunoassay (RIA) was used to determine T levels (nanograms per millilitre of plasma), using the protocol described by Wingfield & Farner (1975) with minor modifications. Two thousand counts per minute (20 μl) of tritiated T were added to each ethanol sample (1 ml) to calculate recovery rates. Samples were left to equilibrate overnight at 7 °C, after which they were transferred to Extrelute columns (0.6 g). Samples were extracted using 6 ml diethyl ether/petroleum benzine (1 : 1 vol), dried under nitrogen and reconstituted in 1 ml of 2% ethylacetate in 2,2,4-trimethylpentane (iso-octane). Reconstituted samples were transferred to short diatomaceous earth chromatography columns to elute the T fraction. T was eluted using 20% ethylacetate. The T fraction was dried under nitrogen and reconstituted in 600 μl phosphate-buffered saline with glucose, pH 7.1; of this 150 μl was used for recoveries and 200 μl in duplicate for the RIA, using hormone-specific antibodies (T3-125; Endocrine Sciences, Calabasas Hills, CA, USA). Assay sensitivity was *ca.* 0.042 ng ml^{-1}. The average recovery rate was 52.5% and the intra-assay coefficient of variation was 8.8%.

(d) *Data analyses*

Observations were related to stage of breeding cycle, i.e. the laying of the first egg (day 0). Statistical analyses were performed using SPSS v. 11.0 (SPSS 2001). Before analysis, values for T level and CP were log-transformed to normalize the data. T levels and CP sizes were analysed using a univariate general linear model, excluding all non-significant terms and their interactions in the order of their significance. Observations involved different birds, i.e. no repeated measures on the same individuals. Means are expressed with standard errors, probability values are two-tailed and the level of significance was set at $p < 0.05$.

3. RESULTS

(a) *Influence of challenges, breeding cycle and male status on testosterone levels and cloacal protuberance sizes*

Out of the captured birds, seven were caught without a challenge and 44 with a challenge (34 caught during a challenge, 10 caught in-between consecutive challenges). The challenge time varied from 7.5 min to 170.0 min, with a mean of 28.7 ± 5.3 min ($n = 44$). The handling time varied from 1.5 min to 10.5 min, with a mean of 3.9 ± 0.3 min ($n = 51$). The use of a challenge did not affect T levels (mean T-level (nanograms per millilitre) without challenge: 1.29 ± 0.22 ($n = 7$); with challenge: 1.17 ± 0.09 ($n = 44$); $F_{1,50} = 0.20$, $p = 0.66$). Furthermore, individual T levels of all males combined were not affected by the challenge time ($r^2 = 0.01$, $n = 44$, $p = 0.65$), or the handling time ($r^2 = 0.02$, $n = 51$, $p = 0.32$). When only primary males were taken into account, T levels remained unaffected by the challenge time ($r^2 = 0.06$, $n = 30$, $p = 0.20$) and the handling time ($r^2 = 0.004$, $n = 36$, $p = 0.72$). The above relationships remained non-significant when different periods of the breeding cycle were tested separately; i.e. before egg laying (when basal T levels were elevated) or after egg laying (i.e. incubation and food provisioning period), all p-values more than 0.59. For the remainder of the analyses the T level data of all birds were pooled.

We used male age, male status, time relative to egg-laying and the interaction between status and time relative to egg-laying as predictors for T levels. Status was not correlated with age (mean age in years; primary males: 2.2 ± 0.4 ($n = 34$) versus subordinate males: 1.8 ± 0.2 ($n = 13$); $t = 0.66$, d.f. = 45, $p = 0.47$). Age was not significant and, after removing this factor, status, days before and after egg laying, and the interaction between status and time relative to egg laying were highly significant (table 1*a*). The T levels of primary males were significantly higher during the period before egg laying compared with after egg laying. By contrast, T levels of subordinate males were low and independent of the stage of breeding cycle (figure 1*a*). In the period before egg laying, T levels of the primary males were significantly higher than T levels of subordinates. After egg laying, T levels of primary males and subordinates did not differ significantly (figure 1*a*).

Only male status was found to be associated with CP sizes (table 1*b*). There was no effect of age on CP size. In contrast to the results for T levels, days from egg laying and the interaction between status and time relative to egg laying had no significant effect on CP size (table 1*b*). For the males measured for CP size, status was not correlated with age (mean age in years; primary males: 1.8 ± 0.4 ($n = 32$) versus subordinate males: 1.8 ± 0.2 ($n = 14$); $t = 1.01$, d.f. = 44, $p = 0.92$). Throughout the breeding cycle primary males had 2.4 times larger CPs than

Table 1. Factors influencing (*a*) the T levels and (*b*) CP sizes of adult male Seychelles warblers. Variables highlighted in bold were left in the minimal adequate model after stepwise removal of non-significant variables.

	T level	
(*a*) independent variables	*F*	*p*
status	**8.811**	**0.005**
age	0.041	0.840
days from egg laying	**8.614**	**0.005**
status × days from egg laying	**6.325**	**0.016**
d.f. = 1,46; $r^2 = 0.306$		

	CP	
(*b*) independent variables	*F*	*p*
status	**17.069**	**0.001**
age	1.164	0.287
days from egg laying	1.712	0.198
status × days from egg laying	0.939	0.340
d.f. = 1,45; $r^2 = 0.347$		

subordinate males (mean size in cubic millimetres; primary males: 39.7 ± 3.8 versus subordinate males: 16.9 ± 3.7; figure 1*b*). Thus both early and late in the breeding season, the CP size of primary males were large whereas the CPs of subordinate males were relatively small (figure 1*b*). Furthermore, despite the fact that for primary males the time relative to egg laying had a significant effect on T levels but not on CP sizes, there was a significant positive correlation between T levels and CP sizes for primary males ($r^2 = 0.16$, $n = 29$, $p = 0.03$) but not for subordinate males ($r^2 = 0.09$, $n = 11$, $p = 0.30$).

(b) *Influence of breeding cycle, extra-group paternity opportunities and cuckoldry risk on testosterone level and cloacal protuberance in males*

Because of the significant effect of male status on T levels and CP sizes, primary and subordinate males were analysed separately. Only days relative to laying influenced primary male T levels (table 2*a*), whereas the factors EGP opportunity and the interaction between EGP opportunity and relative days from egg laying did not explain variation in T levels (table 2*a*). Indeed, there was no clear effect of the presence of fertile neighbouring females on T levels in either the period before or after egg laying (figure 2*a*). In contrast to T levels, CP sizes of primary males were not influenced by the breeding cycle but were influenced by EGP opportunity (table 2*b*). There was a significant positive association between the presence of fertile neighbouring females on CP size in either period (figure 2*b*). Furthermore, the interaction between EGP opportunity and relative days from egg laying did not explain variation in CP sizes (table 2*b*). CP size remained high for up to *ca*. 20 days after egg laying after which it decreased (figure 1*b*; mean (cubic millimetres) before egg laying: 49.2 ± 7.5 versus after egg laying: 36.0 ± 4.2; $t = 1.61$, d.f. = 30, $p = 0.12$). For subordinate males, there was no effect of fertile neighbouring females on T levels ($F_{1,10} = 2.22$, $p = 0.170$) nor on CP sizes ($F_{1,10} = 1.52$, $p = 0.250$) during the period after egg laying (figure 2). Sample sizes for subordinate males in the period before egg laying were too low for analysis (T level: $n = 2$; CP size: $n = 3$).

In the above analyses, cuckoldry risk was not taken as a factor, because cuckoldry risk is absent after egg laying. The effects of cuckoldry risk and EGP opportunity on T levels and CP sizes of primary males were analysed during the period before egg laying only. Cuckoldry risk varied from one ($n = 2$), two ($n = 2$), three ($n = 3$) to four ($n = 2$). T levels were not influenced by either EGP opportunities ($F_{1,8} = 0.54$, $p = 0.54$) or cuckoldry risk ($F_{1,8} = 0.49$, $p = 0.50$). The variation in CP sizes was influenced by EGP opportunities ($F_{1,8} = 6.04$, $p = 0.049$), but not by cuckoldry risk ($F_{1,8} = 1.80$, $p = 0.23$). These results demonstrate that cuckoldry risk had no effect on either T levels or CP size.

4. DISCUSSION

(a) *Testosterone and extra-pair copulations*

It has been argued that in species with high and variable opportunities for EPC, male T levels should correlate with the number of fertile females (Chandler *et al.* 1994; Raouf *et al.* 1997; Van Duyse *et al.* 2000, 2002; de Ridder *et al.* 2000; Peters *et al.* 2001). In a study on the superb fairy-wren, a positive correlation between male T levels and the number of fertile females present in the population was shown (Peters *et al.* 2001). Thus, a rise in the potential for EGP can stimulate a rise in T in individual fairy-wren males. In the Seychelles warbler, we found no correlation between the presence of fertile neighbouring females and T levels for either subordinate males or primary males. Nevertheless, male warblers appear to be perfectly aware of the fertility status of neighbouring females as their intrusions into neighbouring territories coincide with the fertile period of the resident primary female (Komdeur *et al.* 1999). Also, because warblers breed in small territories, males can easily monitor neighbouring females and obtain cues about their fertility status of females from specific vocalizations uttered by females (Komdeur *et al.* 1999). We found a positive significant relationship between primary male CP size (and, therefore, sperm storage capacity) and the presence of fertile neighbouring females irrespective of the fertility status of the males' own female partner. We argue that in the Seychelles warbler the relation between CP size and EGP opportunities—in the absence of correspondingly high T levels—is because of the surreptitious nature of EGC behaviour in this species. Unlike the superb fairy-wren, in which males perform elaborate courtship to obtain EPCs (Green *et al.* 1995), Seychelles warblers gain EGCs by sneakily intruding on other territories without being seen by the resident male, and the soliciting of EGCs does not involve aggressive or displaying behaviour (Komdeur *et al.* 1999). Therefore, in this species T may play an important role in sexual courtship displays towards their own mates, but not in the non-aggressive solicitation of EGCs. However, T might play an indirect role in obtaining EGCs as it may induce the initial growth of the CP (CP size was positively correlated with T levels). Thus, we suggest, that EGC behaviour in the Seychelles warbler is linked with CP (and thus sperm

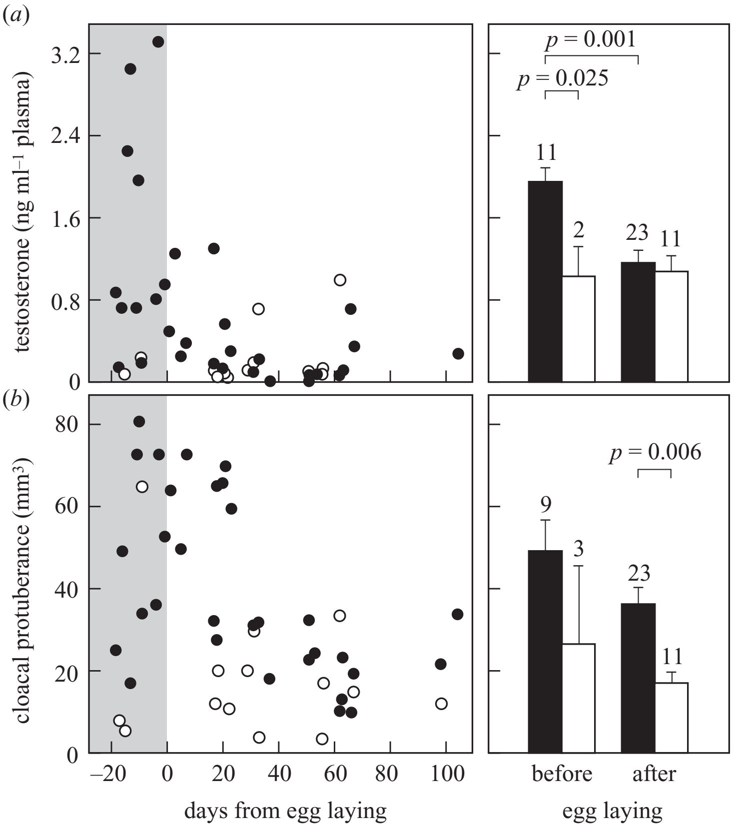

Figure 1. (*a*) T levels and (*b*) CP sizes, of primary (filled circles) and subordinate (open circles) adult male Seychelles warblers in relation to breeding phase. Each point represents an individual. The average T level and CP size of primary males (filled columns) and subordinate males (open columns) before and after egg laying are presented. Bars indicate mean ± s.e.m. and figures indicate sample sizes. Only significant differences between groups are indicated.

Table 2. Factors influencing (*a*) the T levels and (*b*) CP sizes of primary male Seychelles warblers. Variables highlighted in bold were left in the minimal adequate model after stepwise removal of non-significant variables.

	T level	
(*a*) independent variables	*F*	*p*
days from egg laying	**15.577**	**0.001**
EGP opportunity	1.740	0.193
EGP opportunity × days from egg laying	2.280	0.142
d.f. = 1,33; $r^2 = 0.439$		

	CP	
(*b*) independent variables	*F*	*p*
days from egg laying	0.284	0.597
EGP opportunity	**27.452**	**0.001**
EGP opportunity × days from egg laying	0.342	0.563
d.f. = 1,31; $r^2 = 0.489$		

storage), but not directly to T levels. This has the advantage that T levels can decline after egg laying, so that T does not hamper parental care (Peters *et al.* 2002), but males are still able to perform EGCs (Magrath & Komdeur 2003). In the Seychelles warbler, the amount of EGP was independent of T levels and similar in the period before egg laying (with high T level) and in the period after egg laying (with low T level; C. Eikenaar, D. S. Richardson and J. Komdeur, unpublished data). Some males obtained EGP over greater distances than taken into account using our measure, in some cases close to the entire range of the island (Richardson *et al.* 2001) though this was uncommon. In the superb fairy-wren, extra-territorial forays lasted longer for T-treated males than control males, suggesting a role of T in regulating extra-territorial forays (Peters 2002). It might well be that the distance range of EGP in the Seychelles warblers is mediated by T level.

(b) *Testosterone: sexual and aggressive behaviour*

In contrast to migratory bird species, many tropical birds aggressively defend their territories year-round while keeping plasma T levels at a basal level (Dittami & Gwinner 1990; Wingfield *et al.* 1991, 1992; Levin & Wingfield 1992; Wikelski *et al.* 1999). For tropical birds it has been suggested (Wingfield *et al.* 1990, 1997) and shown (Wingfield *et al.* 1991, 1992; Wingfield & Lewis 1993; Levin 1996) that T levels are not influenced by aggression *per se*. Instead, it has been suggested that T is important in mediating the males reaction to his partner's fertility (i.e. mate-guarding and courtship display behaviour (Moore 1984; Hau *et al.* 2000)). Several lines of evidence from our study suggest that in the Seychelles warbler, T is associated with sexual behaviour (i.e.

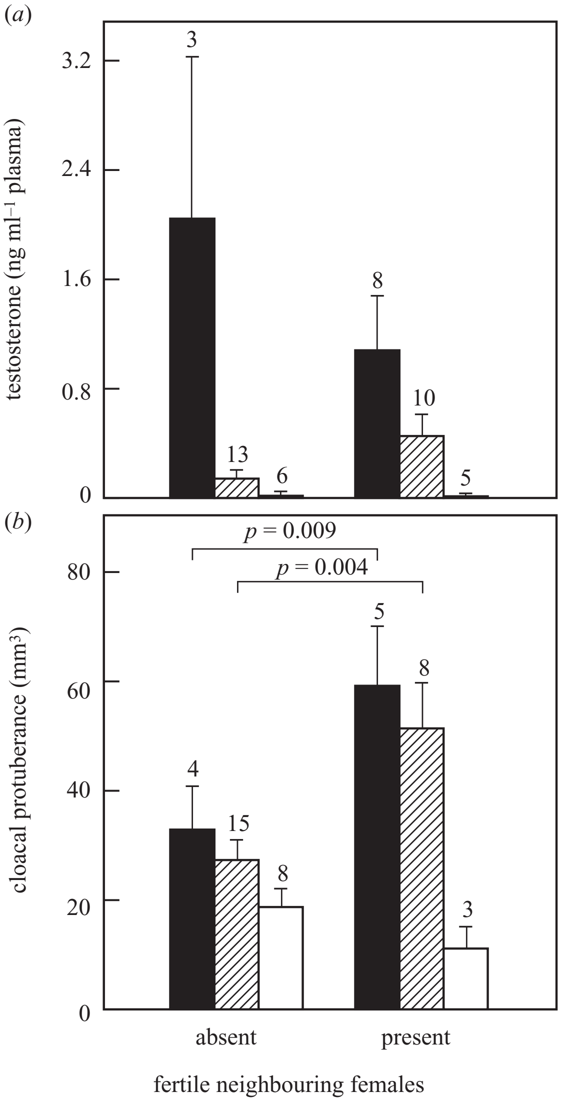

Figure 2. The influence of EGP opportunity (the presence of fertile neighbouring females) on (*a*) T level and (*b*) CP size of primary male Seychelles warblers before (black columns) and after egg laying (striped columns), and of subordinate males after egg laying (white columns). Bars indicate mean ± s.e.m. and figures indicate sample sizes. Only significant effects of EGP opportunity are indicated.

courtship and mate-guarding behaviour), and not with aggressive behaviour.

(i) Aggressive response to a challenge (conspecific male song) did not affect T levels. In other species such agonistic interactions typically elevate T level within minutes (Wingfield & Wada 1989; Wingfield & Soma 2002), which may then promote readiness for further agonistic behaviour in a positive feedback system (the 'challenge hypothesis'; Wingfield *et al.* 1990).

(ii) T levels were independent of cuckoldry risk, despite the fact that male aggression significantly increased with cuckoldry risk (Komdeur 2001).

(iii) Primary male T levels were high before egg laying, when males exhibit most intensive courtship behaviour (Komdeur *et al.* 1999), and decreased rapidly after egg laying when the males still vigorously defend territories.

(iv) Subordinate males that have significantly lower T levels than primary males, do not display sexual behaviours (Komdeur *et al.* 1999; Richardson *et al.* 2001), but participate as much in territorial defence during the breeding season as primary males (Komdeur & Edelaar 2001).

(c) *Testosterone: sexual inactivity in subordinate males versus sexual activity in primary males*

In cooperatively breeding species, adult subordinate males are often reproductively less active than adult primary males (e.g. Reyer *et al.* 1986; Koenig & Mumme 1987; Marzluff & Balda 1990; Schmidt *et al.* 1991; Schoech *et al.* 1991; Russell & Rowley 1993; Poiani & Fletcher 1994). Several hypotheses have been put forward to explain the sexual inactivity of subordinates.

(i) Delayed maturation (Brown 1987). This is not the case in the Seychelles warbler, because males can become primary males at the same age as subordinate males.

(ii) Behavioural suppression, such as intensive mate guarding by the primary male (Brown 1978; Mumme *et al.* 1983; Emlen & Wrege 1986; Reyer *et al.* 1986; Mays *et al.* 1991; Schoech *et al.* 1996*a*). Intensive mate guarding by the primary male does occur in the Seychelles warbler (Komdeur *et al.* 1999), but this does not suggest that subordinate males should have no EGP, nor does it give a reason why only adult males gain EGP.

(iii) Physiological suppression (Wingfield *et al.* 1991; Schoech *et al.* 1996*b*) or lack of appropriate stimuli (Poiani & Fletcher 1994; Schoech *et al.* 1996*a*). In the Seychelles warbler subordinate males have never been seen to be active in courtship behaviour. Therefore, subordinates might not produce adequate levels of T hormones to render them reproductively competent because of hormonal suppression by the primary male (Wingfield & Moore 1987; Wingfield & Farner 1993). However, experimentally augmented T in male subordinates of the azure-winged magpie (*Cyanopica cyanus*) did not affect the likelihood of subordinates becoming breeding males (de la Cruz *et al.* 2003).

In the Seychelles warbler, subordinates had lower levels of T during the fertile female period than did primary males. Although this is based on a rather small sample size, it is consistent with findings of other species (Florida scrub jay, *Aphelocoma c. coerulescens* (Schoech *et al.* 1991, 1996*a*); white-browed sparrow weaver, *Plocepasser mahali* (Wingfield *et al.* 1991); and bell miner, *Manorina melanophrys* (Poiani & Fletcher 1994)). In other species, sexually active subordinate males exhibit T levels equivalent to those of primary males (pied kingfisher, *Cerile rudis* (Reyer *et al.* 1986); harris hawk, *Parabuteo unicinctus* (Mays *et al.* 1991); and Australian magpie, *Gymnorhina tibicen* (Schmidt *et al.* 1991)). However, low T levels in subordinate males may also have an adaptive value if they obtain major benefits from staying in the territory when philopatry is imposed by environmental constraints (Schoech *et al.* 1991). This requires facultative delayed maturation to avoid aggression from the dominant male (Schoech *et al.* 1991). This might be the case for the Seychelles warbler where subordinate males remaining on the parental territory have significant higher lifetime reproductive success than males leaving the natal territory and becoming floaters (Komdeur & Edelaar 2001).

However, instead of lowered T levels in subordinates, T levels of primary males could be elevated by selective attention of the females directed towards the primary males rather than the subordinate male. However, this is not the case in the Seychelles warbler because sexual interactions were often initiated by the primary male, and subordinates were never observed in courtship behaviour (Komdeur *et al.* 1999). In addition, experimental manipulation of T levels in the superb fairy-wren, a species with a comparable social system to that of the Seychelles warbler, clearly showed that T induced courtship behaviour by subordinate males towards their own female (Peters 2002). We clearly need an experimental study to determine whether low T level in subordinates is due to the hormonal suppression by primary males, for example through the removal of primary males from the breeding territory followed by an intensive monitoring of the subordinate's subsequent sexual behaviour and reproductive success.

(d) *Final conclusions*

We conclude that in the Seychelles warbler, T plays a role in sexual courtship displays and mate guarding, but either a minor, or no role, in gaining EGCs in primary males. CP but not T remained elevated after egg laying by their mates, and CP but not T was positively related to the opportunities for EGP. However, we cannot fully rule out a role for T in gaining EGP. For example, male dark-eyed juncos (*Junco hyemalis*) with high T levels gain more EPCs, because females were more attracted to males with high T levels (Raouf *et al.* 1997). Likewise, Seychelles warbler females seeking EGP may sample males before their own fertile period, i.e. during the T peak of neighbouring males.

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