

Female canaries produce eggs with greater amounts of testosterone when exposed to preferred male song

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Abstract

Male birdsong has a great influence in the stimulation of female reproduction. However, female physiological responsiveness to song may depend on the degree of complexity of male song. This is expected because females of iteroparous organisms may increase their fitness by matching their reproductive investment to the predicted value of each reproductive attempt. To the extent that the expression of male ornaments is a signal of male quality, we expect females to increase their investment when paired to highly ornamented males. However, female investment may be cryptic and difficult to detect, such as androgen content in the eggs. In this study, we exposed female canaries (*Serinus canaria*) to attractive and unattractive song repertoires using a crossover design. As predicted, females invested greater concentrations of testosterone in their eggs when exposed to attractive repertoires than when exposed to unattractive repertoires. This implies that song repertoires convey important information about the reproductive value of a given male and suggests that testosterone deposition in egg yolk may be costly.

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Introduction

The stimulatory effect of male song in the reproductive physiology and behavior of female songbirds depends on several factors. At the one extreme, sexual response to heterospecific song is lower than that to conspecific song (Bentley et al., 2000). Within the same species, variation in the expression of song characteristics like repertoire size, song length, etc. also affects female reproduction. For instance, it has been shown that sexual response is stronger to longer songs (Wasserman and Cigliano, 1991), larger repertoires (Kroodsma, 1976; Searcy, 1984) and songs containing particular elements (Vallet et al., 1998). Most of this research has focused on behavioral rather than physiological responses (but see Bentley et al., 2000), but there are reasons to expect that physiological stimulation may also be affected

in the same direction. Numerous endocrine mechanisms are at the base of phenotypic plasticity and may constraint or facilitate adaptive evolutionary responses (Jacobs and Wingfield, 2000; Ricklefs and Wikelski, 2002).

In the context of sexual selection, it is expected that an individual's investment in a given reproductive attempt will be a direct function of the attractiveness of the mate that the individual is paired to (Burley, 1988). The hypothesis follows the optimality logic of parental investment theory (Trivers, 1972) for the case where the value of a given reproductive attempt is predicted by mate attractiveness. Also, if increased investment in the offspring is traded-off with lower survival in the parent, differential allocation will represent a life history trade-off between current reproduction and survival (Stearns, 1992).

The importance of differential allocation in a given species is proportional to the amount of variance in reproductive value that is explained by mate attractiveness in that species (Sheldon, 2000). In species where females are the choosy sex, we expect differential allocation to have a greater relevance in female than in male reproductive investment.

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There is a second way of looking at differential allocation: it can be considered a fine-tuned reproductive adjustment after pairing has taken place. In the real world, female choice of mate does not necessarily correspond to optimal female preference (Wiegmann et al., 1996). Constraints in the opportunities for male sampling and competition between females often result in females choosing males below the standards of their preference. In iteroparous species, females may compensate for this by reducing their reproductive investment with a given suboptimal male to increase the probability of survival and future reproductive attempts with a better male. For instance, waterfrogs *Rana spp.* have been shown to produce fewer eggs when amplexed by hybrid males (Reyer et al., 1999). In this sense, differential allocation can be considered a form of cryptic female choice.

Female reproductive investment in birds can be measured in multiple ways. The first tests of differential allocation looked at obvious aspects of maternal investment such as clutch size or general reproductive output (Sheldon, 2000). However, it has been recently found that there are more subtle biases in maternal investment and that these effects can be present even before laying (Gil et al., 1999).

The avian egg is a complex environment that is nurtured by the mother before ovulation. Several egg components such as the amount of proteins, lipids and antioxidants are known to affect the fitness and development of the offspring (Møller et al., 2000; Nager et al., 2000). Increasing attention is being paid to other, more subtle components of the egg that are passed from the mother to the yolk. An important group of these components is androgens, chiefly testosterone (T). Increasing amounts of T in the yolk are positively correlated with chick vigor, begging intensity and muscle development at hatching, as well as positively influencing social dominance once the bird is adult (Lipar and Ketterson, 2000; Schwabl, 1993; Schwabl, 1996b). However, also negative effects have been found (Sockman and Schwabl, 2000), suggesting that there is an optimum concentration. Females might vary the deposition of androgen within a clutch and in this way affect the outcome of sibling rivalry (Eising et al., 2001). A previous study in the zebra finch (*Taeniopygia guttata*) showed that females deposited more androgens in the eggs fathered by attractive males (Gil et al., 1999). Although these data are consistent with the hypothesis that androgens are a costly investment for the female, further research should address directly this point.

In the present study, we tested whether female canaries (*Serinus canaria*) deposited larger amounts of androgens to their eggs when exposed to preferred attractive male songs. The attractive and unattractive song stimuli that we used were defined by a whole range of attributes that are sexually selected in the song of male canaries and other species. Thus, attractive songs were longer, had a greater repertoire and contained special song phrases, which are preferred by female canaries (Vallet and Kreutzer, 1995). Our aim was not to pinpoint which characteristic is chiefly responsible for

female preference, but to ascertain whether there is a bias in female reproductive physiology dependent on song quality.

Methods

Animals

The experiment involved a total of 24 female common canaries. These birds were hatched in our laboratory from randomly formed pairs coming from several pet shops in an attempt to obtain outbred individuals not belonging to any given artificially selected strain. The experiment was approved by the local Animal Experimentation Committee and met the requirements of French animal legislation.

Experimental design

There were two groups of experimental females with a crossover design. The first group of 12 females was exposed to attractive song for their first clutch and to unattractive song for their second clutch. A second group of 12 females received the treatments in the reverse order. All females were housed individually in cages, without males, and visually isolated from other females. Females belonging to the same experimental group were housed together in soundproof chambers (measuring 80 × 110 × 90). Each soundproof chamber held six different females. We thus used two soundproof chambers per group. Birds were transferred on Day 1 from the single-sex collective cages where they were kept in a photoperiod of short days (10:14-h light–dark) to the individual experimental cages in a photoperiod of long days (14:10-h light–dark). Ten days were allowed for the birds to get used to their new cages. Birds were given nesting material (short cotton threads) on Day 10 at the same time that the experimental treatment started. Song tapes were played daily for 6 h, starting an hour after lights were switched on until all birds had laid their first clutch. A resting period of a week without playback was allowed before the second clutch. Then, nests were emptied of nesting material and song tapes were played again following the same schedule. Nests were inspected daily. Laid eggs were removed and substituted by plastic eggs before incubation. Eggs were then weighed and frozen for further androgen assays.

Song stimulation

The stimuli used in the experiment were built by editing natural canary songs, selecting a large repertoire of different phrases and then building new songs by pasting together several phrases. We used Avisoft software (SasLab Pro 3, Raimund Specht) to edit and produce these songs. The original songs came from males previously recorded from the lab stock. We wished to construct song stimuli that would fall in the two extremes of attractiveness for females.

Several characteristics of bird song have been shown to be selected by female birds, such as large repertoire sizes, long songs or high song rates (Gil and Gahr, 2002). In the common canary, songs are composed of phrases, each phrase being a quick repetition of a syllable and females are particularly sexually responsive to a special type of phrase, with a large frequency range and produced at a very high rate known as “A phrases” (Vallet et al., 1998). We build two kinds of songs, attractive and unattractive. These songs differ in repertoire size, length and presence of A phrases and represent extremes in the usual repertoires of canaries:

- (1) Attractive song. Tapes were built of songs of 10 s each. Each song contained eight different phrases, each of them lasting 1 s. Three of these phrases were A phrases. The other five phrases used had been shown to have mild effect in evoking copulation solicitation displays in previous studies conducted in this species (Vallet et al., 1998).
- (2) Unattractive song. Tapes were built of songs of 5 s each. Each song contained four different phrases, each of them lasting 1 s. These phrases were chosen among the less powerful ones at evoking copulation solicitation displays in previous studies conducted in this species, and none of them was an A phrase.

In all tapes, the songs were delivered at the same rate, that is, five songs per minute. This meant having longer intersong intervals for the unattractive songs. Songs were delivered in bouts of 8 min followed by 2 min of silence. To give all groups the same amount of stimulation, birds in the attractive group were exposed to a bout of stimulation for each two bouts in the unattractive group. Although this procedure resulted in all birds being exposed to the same length of stimulation, it had the obvious disadvantage of exposing birds in the attractive group to only half the number of songs than in the other group. However, we hoped that the great differences in song repertoire size, song length and use of A phrases would compensate a possible effect of number of songs. For each of these stimuli, two different replicates were made, using different phrases. As each experimental group was divided in two different soundproof cages, each cage received a different replicate. In the final analyses, replicates were pooled together by group.

Hormone assays

Yolk concentrations of testosterone (T), androstenedione (A_4) and 5 α -dihydrotestosterone (DHT) were determined by radioimmunoassays at the CEBC (CNRS). Yolks were homogenised in 1 ml of distilled water by vortexing with the aid of some glass beads. We took 100 μ l from a further $\times 10$ dilution of the sample for steroid extraction. This was done by adding 3 ml of diethyl ether to the sample,

vortexing for 1 min and centrifuging for 5 min (4°C, 2000 rpm). The ether phase was decanted after snap freezing the tube in an alcohol bath at -30°C and evaporated under a stream of nitrogen. The dried extract was redissolved in 1 ml of phosphate buffer. Tritiated steroids (1000 CPM) (Amersham Pharmacia Biotech Europe, 91898-Orsay, France) were added to the original samples for the calculation of extraction recoveries. This extraction technique is the standard technique used for plasma samples at the CEBC (Mauget et al., 1994) and is simpler than Schwabl's (1993) extraction for avian egg yolks. The rationale for the change in extraction method was that we only needed to extract a small portion of the yolk to assay the concentration and not the whole yolk as in Schwabl's (1993) original study. In a pilot assay using 10 canary yolk samples, we found that T extraction recoveries were greater (paired *t* test, $t = -4.14$, $df = 9$, $P < 0.01$) using the CEBC method ($X = 96.21$, $SD = 4.4$) than Schwabl's (1993) method ($X = 85.07$, $SD = 7.9$). Nevertheless, both methods gave consistent results for T concentrations ($r = 0.94$, $n = 10$, $P < 0.001$).

Specific steroid antibodies were obtained from Dr. G. Picaper (Médecine Nucléaire, CHU, 45900 - La Source, France) for T and from P.A.R.I.S. laboratories (60200-Compiègne, France.) for A_4 and DHT. The rest of the methods follows standard RIA techniques. The intrassay coefficients of variation for T, A_4 and DHT were 7.3%, 8.5% and 7.5%, respectively. The interassay coefficient of variation for T was 8.5%. Only one assay was performed for A_4 and DHT. The lowest detectable quantities for T, A_4 and DHT that were significantly different from zero at a 95% coefficient level were 7.8, 1.9 and 3.9 pg/assay tube, respectively. According to the extraction conditions, this corresponds to concentrations of 12.0, 3.0 and 6.0 pg/mg of canary egg yolk, respectively, for each hormone. Cross reactivity of T antibody was <15% for DHT and <1% for A_4 .

Statistical analyses

The design of the experiment corresponds to a crossover design with repeated measurements (the one to five eggs laid per bird in each clutch). We used the procedure MIXED in SAS (version 8.2), with female nested within sequence as a random effect, and a repeated effect of lay order (1 to 5) within bird treatment period.

The variance-covariance structure for the repeated egg measurements was selected by comparing the results of the different possibilities and choosing the best fit by using the improvement in the AIC criterion as the basis for this determination (Littell et al., 1996). The autoregressive covariance structure AR(1) was found to be a significantly better fit for all measurements. This structure implies that the further apart the eggs are in the laying order, the lower the correlation between their measurements. This is entirely consistent with biological expectations as female physiolog-

Table 1

Concentrations of the three main egg yolk androgens in a subsample ($n = 9$) from the yolks assayed in the study

	\bar{X} (pg/mg)	SEM	Range
Testosterone (T)	80.68	17.53	17.30–142.30
Dihydrotestosterone (DHT)	43.71	6.67	15.90–68.90
Androstenedione (A_4)	10.03	2.32	0.90–21.20

ical condition is expected to change gradually during egg laying.

We compared the models resulting from fitting laying order as a classification variable and as a continuous variable. The classification effect was not a significant improvement over a simple linear regression on laying order (within clutch). Similarly, we tested a quadratic regression component but this was found not to be significant. Thus, the effect of laying order was fitted as continuous lineal variable.

The best fitting, most appropriate model was determined using a combination of F tests, for fixed effects, and AIC, for determining the random effect of female. Additionally, to assess the statistical significance of the latter, we fitted a similar final model, but without the random female effect, and calculated its departure from the main model.

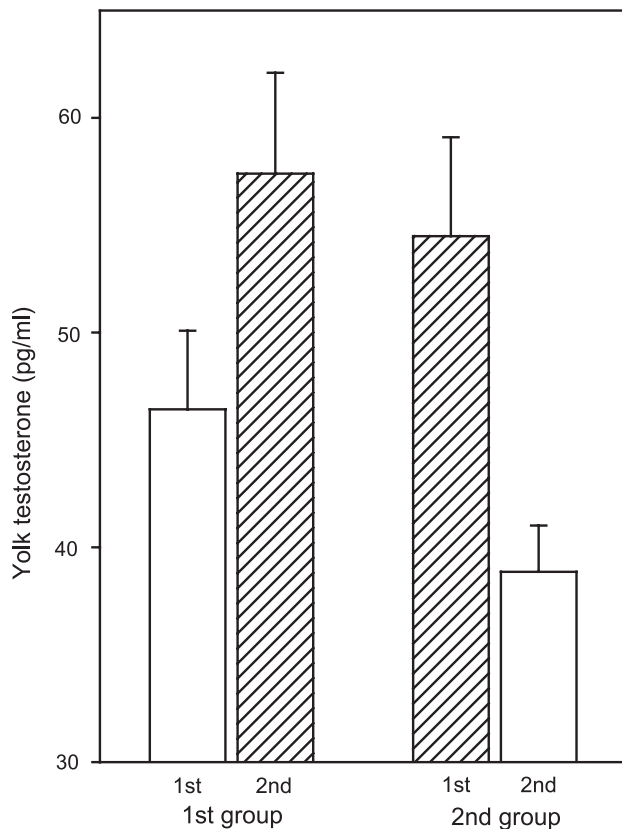


Fig. 1. Androgen concentrations (mean pg/mg yolk \pm SEM) in the eggs produced by females exposed to attractive (hatched bars) and unattractive song (empty bars). Each group of females laid two clutches and the order of treatments was reversed in each group.

Results

Concentrations of T, A_4 and DHT were assayed in a random subsample of nine yolks. The results show very high correlations among these three androgens (T–DHT: $r = 0.943$, $n = 9$, $P < 0.001$; T– A_4 : $r = 0.974$, $n = 9$, $P < 0.001$; A_4 –DHT: $r = 0.910$, $n = 9$, $P < 0.001$). Therefore, we assayed only T in the remainder of the samples because this was the androgen with the highest concentrations of the three (Table 1).

As predicted, song attractiveness had a major influence on the concentration of T in the egg [$F(1,24.4) = 10.39$, $P < 0.001$; Fig. 1] and in the total T content [$F(1,23.3) = 9.88$, $P < 0.01$]: females produced eggs with higher concentrations and total content of T when exposed to attractive song. However, females did not produce significantly heavier eggs or yolks in response to the song stimuli (all $F < 0.82$, NS). The effects of clutch number or sequence were not significant for any of the measurements (all $F < 0.73$, NS). The effects of female were large and significant for all traits, and indeed the repeatability of the traits was quite large (all > 0.40 ; $\chi^2_1 > 6.30$, $P < 0.05$). Neither nest weight, latency to lay, latency to build nor clutch size showed differences between the treatments [all $F(1,21) < 1.8$, $P = 0.19$].

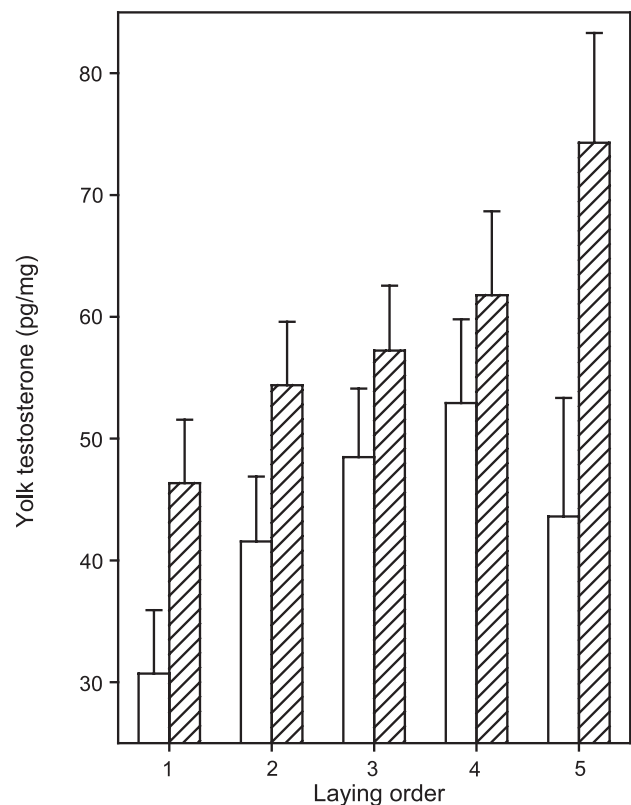


Fig. 2. Mean (\pm SEM) T concentration in eggs with respect to egg laying order for females exposed to attractive (hatched bars) and unattractive song (empty bars).

Laying order was found to influence all egg measurements. With increasing laying order, eggs became lighter [$F(1,92.2) = 14.5$, $P < 0.001$] and had smaller yolks [$F(1,91) = 59.57$, $P < 0.001$]. Conversely, yolk T concentration increased with laying order [$F(1,120) = 19.33$, $P < 0.001$; Fig. 2], as did total T content [$F(1,121) = 6.99$, $P < 0.01$], although yolks got lighter with increasing laying order. We tested whether the pattern of change of each measurement with laying order differed between the treatments by testing the significance of an interaction factor. No statistically significant interaction with treatment was found, and thus the interaction was removed from the model. We also examined whether the difference in T concentration and total T content between the first and the last eggs within a clutch was greater for females exposed to attractive than to unattractive song, but there were no differences between the groups [all $F(1,21) < 0.9$, NS].

We tested the covariance between the several measures of egg quality by performing correlations with the residuals from the model and obtaining female averages. Females that laid heavy eggs also had heavier yolks ($r = 0.71$, $n = 21$, $P < 0.001$), but there was no correlation of egg weight with T concentration ($r = 0.22$, $n = 21$, NS).

Discussion

The results of this study show that the concentration of T in the yolks of eggs laid by female canaries is dependent on the attractiveness of the songs that females are exposed to during egg formation and laying. Females deposited higher concentrations of T in their eggs when they heard attractive male songs than when they heard unattractive songs. There were no differences in the other measures of maternal investment, like clutch size, latency to nest building, egg or yolk weight.

A similar pattern of differential deposition of yolk-T has been found in the zebra finch in response to variation in male attractiveness (Gil et al., 1999). Previous research has shown that yolk-T is a beneficial component of avian eggs, and that growth, begging and dominance in canaries (Schwabl, 1993, 1996b) and muscle development in red-winged blackbirds (*Agelaius phoeniceus*) (Lipar and Ketterson, 2000) are positively affected by high levels of yolk-T. However, one study in kestrels (*Falco sparverius*) has found negative effects of high yolk androgen concentrations (Sackman and Schwabl, 2000), suggesting that there is an upper limit over which the costs may outweigh the benefits and that some species may already be at this maximum limit. There are several possibilities for a cost related to yolk-androgen deposition: androgen-induced immune suppression in the female or the offspring (Folstad and Karter, 1992); excessive sibling aggression that may lead to non-adaptive brood reduction (Mock and Parker, 1997); or an increase in nest predation due to high begging rates (Haskell, 1994). Neither of these hypotheses has yet been examined in any species.

It has been proposed that patterns of differential allocation of yolk-T might be an epiphenomenon of a sex-biasing mechanism in birds (Petrie et al., 2001). Male-biased sex ratios are expected when females pair with attractive males (Burley, 1981), and Petrie et al. (2001) have proposed that females bias their sex ratios through differential levels of androgens. Thus, differences in yolk-T would correspond to biases in sex ratio rather than to differential allocation. This hypothesis is based in the fact that the yolk sac of peacock (*Pavo cristatus*) eggs in their 10 days of incubation contained different amounts of androgens depending on the sex of the embryo (Petrie et al., 2001). A problem with that study is that hormones were assayed after a fairly long incubation time (Day 10 of a 28-day incubation period), and by then, early hormonal activity in the embryo might have modified the original yolk hormonal patterns. There is evidence that androgen receptors are present very early in development (Reid et al., 1981), implying that hormonal levels in the yolk once the embryo is developed may not correspond to levels of maternal origin. A recent research conducted with chickens has found no overall sex differences in egg hormonal contents when these are assayed soon after laying (Müller et al., 2002), although a complex interaction of these factors with female social status was detected. Furthermore, the hypothesis of Petrie et al. (2001) cannot be considered an alternative to the hypothesis of differential allocation as the two hypotheses belong to different levels of analysis (see for example Sherman, 1988). Unless a greater amount of androgens is not equally beneficial to chicks of each sex, whether this greater deposition is consequence of a mechanism designed to bias sex ratio or not is immaterial to the question of differential allocation. If male and female offspring benefit to the same extent of this deposition of hormones, female parents would benefit from the overall higher fitness of their offspring in addition to eventual benefits originating from the adaptive bias in sex ratio.

We did not find differences in the weight of the eggs of the yolks with respect to male attractiveness, contrary to previous evidence reported for the mallard (*Anas platyrhynchos*) (Cunningham and Russell, 2000). Possibly, females are able to relatively rapidly vary their deposition of yolk-T, whereas changes in egg and yolk weight may be slower and require a longer time between exposure to song and laying to take effect. In our experiment, females were not allowed a long acquaintance with the songs, and this may explain why we did not find differences in egg or yolk weight. Furthermore, in the mallard, there is no parental feeding and therefore differences in egg size are bound to be more crucial than in altricial species.

Previous studies have also shown that canaries exposed to conspecific song built nests more readily (Hinde and Steel, 1976), grow larger follicles and produce a larger number of eggs than those exposed to heterospecific song (Bentley et al., 2000). We did not find differences in number of eggs laid in our study. This could be explained if the

determination of clutch size is chiefly related to female quality and not to male attractiveness (Christians, 2002). A previous study also failed to detect significant differences in nest building and laying latencies between canaries exposed to songs containing attractive and unattractive sexy phrases (Leboucher et al., 1998).

The pattern of T deposition within a clutch may influence sibling hierarchies and affect patterns of brood reduction (Schwabl, 1996a). Hatching asynchrony is a common phenomenon in canaries in captivity, whereby late eggs in a sequence hatch one of 2 days later than the rest. As a consequence, a size hierarchy is formed among the siblings and chicks hatching from late eggs will be more likely to die in case of an eventual brood reduction (Mock and Parker, 1997). Patterns of androgen distribution within a clutch can diminish or reinforce the effects of hatching asynchrony, depending on whether androgen increases or decreases with laying order, respectively (Eising et al., 2001). Among the many hypotheses that have been proposed to explain the adaptive value of hatching asynchrony, some propose that higher levels of asynchrony would be adaptive when food supply is predicted to be low or of poor quality (Wiebe and Bortolotti, 1994). Following this hypothesis, we could predict that if song attractiveness is an honest signal of the quality of paternal care in the canary (Hoelzer, 1989), the pattern of androgen distribution within a brood should differ between the treatments. Explicitly, the increase of T with laying order should be steeper for the attractive treatment than for the unattractive treatment. However, we found no differences between the treatments: T increased from the first to the sixth egg in a similar fashion for females exposed to attractive and unattractive song, although a certain trend in the predicted direction was indeed observed (Fig. 2). Neither did we find differences between clutches in the within-brood yolk-T coefficient of variation or in the difference between the first and the last egg. Similar results were found in a previous experiment with zebra finches (Gil et al., 1999).

Bird song is a complex secondary sexual trait that presents multiple characters that can be sexually selected (Gil and Gahr, 2002), such as repertoire size or song rate. The origin and maintenance of song repertoires in songbirds are best explained by mechanisms of sexual selection (Searcy and Yasukawa, 1996), although field evidence shows that in many species, female preference for song repertoires is rather weak (e.g., Gil and Slater, 2000). It has been suggested that other, more revealing, aspects of male quality may override preferences for song repertoires (Searcy and Yasukawa, 1996) in many species. The results of the present study suggest that female preferences may be quite cryptic, as in the form of prelaying androgen deposition in the egg. This mechanism may be used by females to fine-tune their investment after pairing has taken place in situations where a perfect choice cannot be made (Wiegmann et al., 1996) or when other uncorrelated aspects of male quality are primarily used for male choice.

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