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The importance of novelty: Male–female interactions among blue-black grassquits in captivity

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ABSTRACT

Mate choice is a primary mechanism driving the evolution of sexually selected traits such as elaborate displays and ornaments. In a majority of taxa studied to date, females are seen to actively sample and evaluate multiple males, presumably to optimize mating opportunities. During this process females may encounter males both familiar and novel, a distinction that might influence how mate choice proceeds. Using a socially monogamous passerine, the blue-black grassquit (*Volatinia jacarina*), we studied how females respond to novel versus familiar ("paired") males, and how encounters with novel males influence subsequent interactions with their paired males. Additionally, we measured the hormonal response of males after visualizing their paired females interacting with novel males. We found that females were attentive to novel males irrespective of these males' phenotypic attributes, suggesting that in these interactions novelty is highly relevant. After exposure to novel males, females tended to respond aggressively towards their paired males; by contrast, the behaviour of males towards their paired females did not change. Moreover, we did not detect any hormonal responses of males to viewing their paired females interacting with novel males. Together these results suggest that the distinction between familiarity and novelty may hold special relevance for females in mate choice, a finding that bears upon our understanding of the evolution of extra-pair paternity and reproductive behaviour.

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1. Introduction

Mate choice is often considered the principal mechanism of sexual selection, driving the evolution of elaborate mating ornaments and displays (Andersson, 1994; Andersson and Simmons, 2006; Kokko et al., 2006). Yet, for most species we know surprisingly little about the mechanisms that underlie mate choice. Basic information is often still required regarding, for example, which ornaments, colour patterns and behaviours are being assessed, what kinds of information these traits encode, and what benefits animals derive by choosing specific mates. Some long-standing questions about mate choice also concern the strategies females use in mate sampling. As females sample multiple males, how do they compare them and how do they decide which male is the optimal partner (e.g., Beckers and Wagner, 2011)? In this regard, studies in diverse taxa suggest that females sampling mates exhibit some preference for novelty over familiarity (fruitflies: Ödeen and Moray, 2008; crickets: Gershman, 2008; pseudoscorpions: Zeh et al., 1998; and guppies: Hughes et al., 1999). In a recent study on bearded reedlings (*Panurus biarmicus*), for instance, females showed interest in novel males that was independent of their social partners' attractiveness (Hoi and Griggio, 2012). Attending to novel males may enable females to access a wider sample of potential mates. Accordingly, in the presence of unpaired same-sex conspecifics, males may be prompted to increase contact with their mates (e.g., for mate guarding), and perhaps incidentally provide females with indirect benefits (e.g., in parental care).

One way to gain insights into mechanisms of mate sampling is through laboratory studies, which allow precise control of the variables that are presumed to influence mate choice. In birds, studies with captive populations have emphasized the function in mate choice of both visual and acoustic signals, often showing that females prefer males with elaborate sexual traits (Burley et al., 1982; Hill, 1990; but see Widemo and Saether, 1999 and Griggio and Hoi, 2010 for alternative examples). Female house sparrows (*Passer domesticus*), to illustrate, prefer to associate with males with larger melanin-based ornaments (Møller, 1988) and

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larger white wing bars (Moreno-Rueda and Hoi, 2011); female common pheasants (*Phasianus colchicus*) prefer males with longer tails (Mateos and Carranza, 1995); and female swamp sparrows (*Melospiza georgiana*) give more copulation solicitation displays in response to playback of songs of high vocal performance (Ballentine et al., 2004). By contrast, the relative roles of familiarity versus novelty in mate sampling by captive birds has been relatively overlooked.

In passerine birds, we might predict females to be partial to novel males, given high natural rates of extra-pair paternity (EPP) (Griffith et al., 2002). This is because females are thought to derive greater benefits from mating with multiple partners than from mating repeatedly with the same partner (Jennions and Petrie, 2000; Griffith et al., 2002; Eakley and Houde, 2004; Neudorf, 2004; Macedo et al., 2008). The benefits of extra-pair mating for females can be either indirect or direct. Indirect benefits are usually genetic and have been the focus of multiple studies (Kempenaers et al., 1992; Stapleton et al., 2007, but see Ferreti et al., 2011; Lee, 2012). Direct benefits, on the other hand, are not as clearly detected nor easily interpreted, but have been demonstrated in a few studies (Gray, 1997; Townsend et al., 2010). To gain the benefits of extra-pair mating, females must spend time and energy searching for and evaluating extra-pair mates, and may put at risk their social partners' investment in parental care (Petrie and Kempenaers, 1998). The distinction between novel and familiar males may ultimately influence how females balance the costs and benefits of mate selection.

Male behaviour also likely influences female mate selection. Given the high costs associated with loss of paternity, males already paired with females are expected to discourage those females from seeking extra-pair copulations. Male tactics towards this end may include mate guarding (Beecher and Beecher, 1979), decreasing paternal investment in nests with extra-pair young (Weatherhead et al., 1994; Gowaty, 1995), and retaliating aggressively against females that stray (Westneat and Stewart, 2003). In the latter case, aggression and forced copulations have been described for contexts where males are unsure about female fidelity (Johnstone and Keller, 2000; Valera et al., 2003).

Mechanistically, male mating tactics and aggression are regulated in part by steroids, namely testosterone (T) and corticosterone (CORT). Varying circulating levels in these steroids influence male social behaviours including mate guarding, male–male interactions, and responses to territorial challenges, and can also modulate the expression of subsequent behaviours (Wingfield et al., 1990; Oliveira, 2004; Soma, 2006). For example, short exposure to simulated territorial intrusions conducted with the song sparrow, *Melospiza melodia*, led to increased T plasma levels 10 min after the event, and T levels remained elevated for up to 1 h afterwards (Wingfield and Wada, 1989). However, because maintaining high T levels generates various physiological costs, the challenge hypothesis predicts that T levels should be elevated only when needed (Wingfield et al., 1990). CORT is released by the adrenal gland, and plasma concentration in birds typically increases significantly after about 3 min in response to stress (Wingfield and Silverin, 1986; Romero and Romero, 2002). While much is known about how these steroids vary with male–male interactions, less is known about how they might regulate male responses to female behaviour.

Here we studied, in a captive population of blue-black grassquits (*Volatinia jacarina*), female responses to novel versus familiar ("paired") males, as well as male behavioural and physiological responses when their paired females were exposed to novel males. With this study we seek to clarify the factors that influence female readiness to copulate with extra-pair partners, how female behaviour may affect the dynamics of a pair, and how males might respond to females that interact with novel males. First, we asked whether "paired" females show any interest in novel males. Despite their socially monogamous mating system, female blue-black grassquits in nature show high rates of extra-pair mating (Carvalho et al., 2006; Manica et al., unpublished data), which suggests that females in captivity might likewise show an interest in novel males. Second, we asked whether any interest females do show in novel males is influenced by the novel males' body condition and plumage traits, both absolutely and relative to traits of her paired male. We expected females to exhibit interest especially when a novel male was phenotypically superior to the social mate. Third, we assessed social pairs' behaviour following the experimental procedure, when male and female pairs were reunited, after females had been exposed to novel males. We expected that interactions of paired females with novel males would elicit reactions from socially paired males, both physiologically (e.g., increases in T and CORT) and behaviourally (e.g., increased aggression directed towards the paired female).

2. Methods

2.1. Study organism

The blue-black grassquit is a socially monogamous, intratropical migratory passerine. Field observations indicate that breeding territories are tightly clustered in a *lek*-like spatial pattern (Webber, 1985; Almeida and Macedo, 2001). Before the breeding season males moult and acquire an iridescent, structurally coloured blue-black plumage, with peak reflectance in short wavelengths (Maia et al., 2009). Throughout the breeding season, males produce characteristic courtship displays, in which they leap upwards, reveal white wing patches while flapping their wings, and emit short vocalizations (Alderton, 1963; Almeida and Macedo, 2001). In addition to these displays, male reproductive effort includes nest construction and provisioning of young (Alderton, 1963; Almeida and Macedo, 2001). Blue-black grassquits have one of the highest levels of extra-pair fertilization rates in passerine birds, of approximately 50% of nestlings in over 60% of sampled nests (Carvalho et al., 2006). A laboratory study showed that social context relative to group composition (i.e. number of males interacting in a group) affected male testosterone plasma concentration and behaviour (Lacava et al., 2011).

2.2. Study subjects

A total of 120 individuals (60 males and 60 females) were mist-netted within the Campus of the University of Brasilia (15°46′ S, 47°52′ W) in December of 2004/2005 and January of 2005/2006, which is at their peak breeding time. Until the end of June 2005, males and females were kept as separate groups and in visually isolated compartments of an outdoor aviary (3.0 m × 3.0 m × 2.0 m) that was exposed to natural conditions of lighting and temperature. At this time (5 months before experiments began in each year), males and females were paired randomly ($N = 60$ pairs) and placed in individual cages (0.70 m × 0.50 m × 0.40 m) within the outdoors aviary. Cages were visually but not acoustically isolated. Cages were set up side by side with visual barriers between them. Experimental trials were conducted from December to February in 2005/2006 and 2006/2007, months that coincide with the grassquits' breeding season in central Brazil. During the pre-experimental and paired phase, paired males were observed to produce display leaps and vocalizations, and to copulate, indicating that courtship and reproductive activities were developing normally. The additional captured males ($N = 11$: 2005/2006 = 5; 2006/2007 = 6) and females ($N = 6$) were maintained in same-sex shared cages (1.5 m × 3.0 m × 2.0 m), to be presented to paired males and females as "novel" stimuli (see below). All birds were

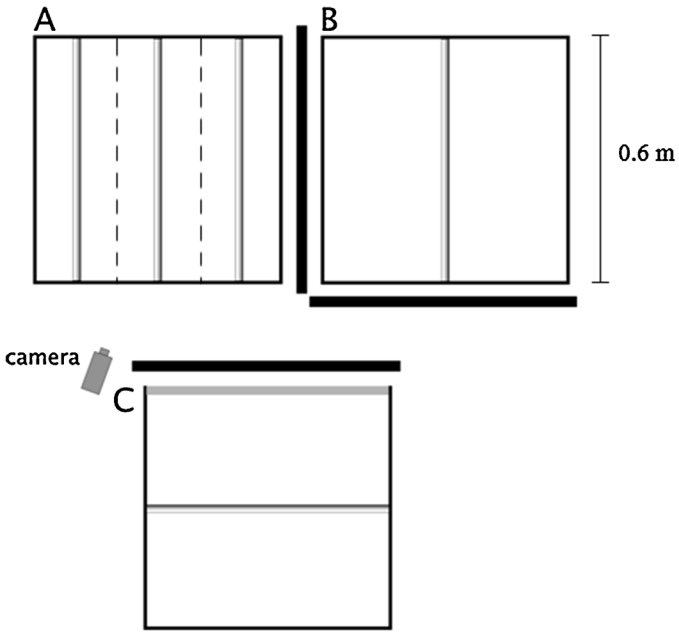

Fig. 1. Schematic diagram of experimental apparatus, overhead view. Cage *A* always housed a "paired" female, and Cage *C* her "paired" male. Cage *B* was either empty or housed a "novel" male or female, depending on the experimental treatment (see text and Table 1). The grey bar in Cage *C* represents one-way glass, the three black bars represent opaque removable barriers, and the five dowels represent perches. The dashed lines in Cage *A* represent the division of the three sections used to evaluate female response.

provided ad libitum access to food (mixture of seeds) and vitamin-supplemented water, and during the breeding season were also provisioned twice weekly with mealworms and a high protein food mixture. The experiments described below were conducted in accordance with the current laws of Brazil, and capturing and banding activities were performed under permit 237 DIFAS/DIREC from the Instituto Brasileiro de Recursos Renováveis – IBAMA.

2.3. Experimental apparatus and treatments

All experimental trials took place within an apparatus (Fig. 1) consisting of three wire mesh cages (0.6 m × 0.6 m × 0.6 m each). Two of the cages, *A* and *B*, were positioned side by side, and the third cage, *C*, was positioned 60 cm in front of the other two, but offset towards Cage *A*. The experiment comprised four experimental treatments. In all four treatments, Cage *A* contained a female ("paired female"), and Cage *C* contained the male with whom she had been paired ("paired male") for 5 months previously. Cage *C* had one-way glass (no mesh) facing the other two cages, with the other cage surfaces covered with black paper. Thus, the paired male inside Cage *C* could not be seen from the outside, but could see out through the one-way glass. Ambient lighting was from standard fluorescent lamps (6 × 30 W), and Cages *A* and *B* were additionally lit with a fluorescent bird lamp (Arcadia Bird Lamp; 2.4% UVB, 12% UVA).

Depending upon the experimental treatment, Cage *B*: (i) was empty ("Control 1"); (ii) contained an unfamiliar female ("Control 2"); or (iii) contained a novel male ("Manipulation 1" and "Manipulation 2"; see Table 1). Before each experimental trial, birds were acclimated to their cages for 20 min. During this period birds were visually isolated from each other with three removable opaque barriers (dark bars, Fig. 1). Trials began with removal of opaque barriers, and lasted 30 min. Previous pilot trials indicated that almost all activities were concentrated within this period. In "Control 1", "Control 2", and "Manipulation 1", two of the three opaque barriers were removed (barrier separating Cages *A* and *B* and barrier in front of Cage *C*). In these treatments the paired male could not view the interior of Cage *B* (i.e. the novel male). In "Manipulation 2", all three opaque barriers were removed, so that the paired male could view both the paired female and the novel male.

Behavioural activities and interactions of birds in Cages *A* and *B* were videotaped, and video images were also monitored from an adjacent room. The white background in Cage *A* was divided into three vertical sections, each of which contained a centrally located perch that allowed us to monitor the time spent by the paired female in each division of the cage. Female response to the Cage *B* stimulus was quantified as the proportion of time spent next to the neighbouring cage, either on the cage floor or on the perch in that section (about 10 cm from neighbouring cage). We decided to use this conservative measure of female response because it is the closest distance an individual of grassquit size could stay relative to the adjacent cage. Additionally, previous studies that evaluated female preference in a mate-choice apparatus considered approximately the same distance (Bennett et al., 1996, see also Wolf et al., 2004). We also documented the paired female's behaviour during each trial, which included copulation solicitations, hops and vocalizations.

For three of the four experimental treatments (all but "Control 2"), a second experimental phase immediately followed the first, wherein the paired male and female were reunited in their home cage, and their behaviour was monitored for another 30 min. We observed interactions during this phase and classified behaviours into one of three categories: agonistic (pecks and chases), neutral, or amicable (copulation solicitation, or pair in close proximity). Each behavioural interaction was identified relative to the sex of the initiator (in the case of pecks, chases, copulation solicitations). Additionally, we recorded the amount of time spent by the birds in close proximity, i.e. <10 cm separation.

2.4. Hormone sampling and assays

For 10 males in "Manipulation 1" and for 10 males in "Manipulation 2", we extracted blood samples within 2 min after the first experimental phase had ended, before birds were placed into the second experimental phase. Blood was collected (average of 50 μl) in heparinized microcapillary tubes, and centrifuged for plasma separation, at 11,500 revolution/min for 5 min (~14,000 G). Blood plasma was stored at −20 °C, and subsequently analyzed for hormone levels using radio-immunoassays. The free fraction of steroids was extracted from plasma samples with diethyl ether as in Scott and Vermeirssen (1994). Steroid residues were re-suspended in 1 ml assay buffer and stored again at −20 °C until assayed T and CORT. The antibody for T was purchased from Research Diagnostics (RDI-TRK2T2, Concord, USA), and the antibody for CORT from Sigma–Aldrich (C8784, Saint Louis, USA). For each hormone all samples were assayed in a single run; intra-assay coefficients of variation for T and CORT were 2.4% and 1.3%, respectively. Despite running the whole procedure, we failed to obtain hormonal levels for some individuals due to small plasma volume (see sample sizes in the result section).

2.5. Body and plumage traits

After experimental trials were complete we took the following measurements from both paired and novel males: (1) length of right tarsus and wing, and beak and tail to the nearest mm; (2) mass to the nearest g; and (3) percent coverage of nuptial plumage, scored on a scale from 0 to 100%.

We also collected 4–5 feathers with forceps from each of three body parts (head, back and breast), and evaluated their spectral reflectance through spectrophotometry (Ocean Optics USB4000;

Table 1
Summary of the experimental design.

Experiment	Treatment	*N* pairs	Pair reunited	Hormone assays
Control 1	Cage *B* empty	10	Yes	No
Control 2	Cage *B contains female*	3	No	No
Manipulation 1	Cage *B contains novel male*: Paired male cannot see novel male	20	Yes	Yes ($N = 10$ males)
Manipulation 2	Cage *B contains novel male*: Paired male can see novel male	10	Yes	Yes ($N = 10$ males)

200–1100 nm range; 3648 pixels) under a pulsed xenon light source (Ocean Optics PX-2; 220–800 nm range). All measurements were taken relative to a WS-1-SS diffuse reflectance white standard (Ocean Optics). We took readings using a bifurcated fibre-optic probe, mounted on a probe holder that excluded all ambient light and where incident and reflected light angles were equal (45°). Reflectance data were calculated for the three body parts using values for brightness, intensity, hue, and blue and UV chroma (Montgomerie, 2006). Brightness ($R_{320–700}$), or sum of total light reflectance, was calculated as the sum of percent reflectance values from 320 nm to 700 nm. Colour intensity was defined as the maximum reflectance reached (R_{max}). Hue is the main colour reflected by the feather, calculated as the wavelength of maximum reflectance (λR_{max}). Chroma, a measure of spectral purity, is the ratio between total reflectance in the range of interest and total reflectance across the entire spectrum. UV chroma was calculated as the ratio between UV reflectance ($R_{320–400}$) and total reflectance ($R_{320–700}$), and blue chroma as the ratio between blue reflectance ($R_{400–500}$) and total reflectance ($R_{320–700}$). We considered this band of wavelengths for the blue chroma based on molecular evidence about cone sensitivity available from other species (Hart et al., 2000; Ödeen and Håstad, 2010).

We calculated the repeatability (r) for spectral measures (see Lessells and Boag, 1987), and found generally high and statistically significant repeatabilities (all $P < 0.001$): (a) head, brightness $r = 0.74$ ($F_{50,233} = 13.74$), intensity $r = 0.72$ ($F_{50,233} = 12.74$), hue $r = 0.69$ ($F_{50,233} = 11.42$), UV chroma $r = 0.32$ ($F_{50,233} = 2.93$), and blue chroma $r = 0.52$ ($F_{50,233} = 3.93$); (b) back, brightness $r = 0.74$ ($F_{50,233} = 11.94$), intensity $r = 0.75$ ($F_{50,233} = 14.73$), hue $r = 0.53$ ($F_{50,233} = 6.26$), UV chroma $r = 0.86$ ($F_{50,233} = 8.00$) and blue chroma $r = 0.91$ ($F_{50,233} = 10.45$); (c) breast, brightness $r = 0.72$ ($F_{50,233} = 16.42$), intensity $r = 0.75$ ($F_{50,233} = 14.61$), hue $r = 0.44$ ($F_{50,233} = 4.66$), UV chroma $r = 0.89$ ($F_{50,233} = 16.54$), and blue chroma $r = 0.86$ ($F_{50,233} = 6.44$).

To facilitate the statistical analysis of reflectance, we performed a principal component analysis (PCA) on average reflectance values measured for the three body regions (Table 2). The first principal component (PC1) explained 50% of total variation, and showed a strong and positive association with brightness and intensity. The second principal component (PC2) explained the remaining 25% of the total variation, and showed strong loadings for chroma (a positive loading for UV chroma, and a negative loading for blue chroma). Thus, birds with a high PC1 score were brighter and more intensely coloured (i.e. higher levels of maximum reflectance), and birds with a high PC2 score showed a higher spectral purity in the UV range and lower scores in the blue range.

Table 2
Eigenvectors for the two first principal components of a principal components analysis (PCA) on five colour variables for three body parts of the blue-black grassquit.

Variable	PC1	PC2
Brightness	0.950	0.133
Intensity	0.896	0.283
Hue	0.698	0.243
Blue chroma	0.309	−0.794
UV chroma	−0.456	0.668
Variance explained (%)	49.90	24.69

2.6. Statistical analyses

To assess whether paired females showed interest in novel males, we tested for variation in the time they spent in the different cage divisions, using a one-way repeated measures ANOVA due to the fact that the same females evaluated different males during the experimental trials. We used Mauchly's test to evaluate the sphericity assumption and whenever it was violated using the appropriate correction. Degrees of freedom for repeated measures ANOVAs were corrected using Greenhouse–Geisser estimates of sphericity. Whenever we found a significant result in the repeated measures ANOVA we conducted paired t tests to compare pairs of levels of the independent variable (i.e. positions within the cage), and then applied a Bonferroni correction.

To evaluate if female interest was associated with phenotypic traits of novel males, we first calculated Pearson correlations between traits of novel males and the time females spent in proximity to novel males (i.e. time spent in the third of the cage closest to the novel male). For these calculations, male phenotypic traits included raw trait values, a composite measure of male traits, and an index of male body condition. Composite measures of traits for each male were generated using Principal Components Analysis (PCA), with PC1 considered an index of total body size. The index for male body condition was calculated using the residuals of linear regressions between body mass and PC1 for each male. We then calculated a second set of correlations between female location within her cage and male traits (as above, raw values, PCA, and body condition), but this time on a relative scale, i.e. as differences in trait values between a female's paired mate and the novel male she was presented with.

To evaluate whether male behaviour and hormone levels differed between Manipulations 1 and 2 (paired male could or could not view the novel male, respectively), we used Mann–Whitney U and t tests. Finally, we evaluated whether presentation of novel males influenced the subsequent aggressive behaviour of reunited paired males and females, using Mann–Whitney U and t tests and Fisher's Exact tests (comparing Control 1 versus Manipulations 1 and 2).

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., 2004). Data were tested for normality with a Shapiro–Wilk test and non-parametric tests were used when necessary (as indicated above).

3. Results

3.1. Behaviour of paired females

In both control treatments, "paired" (Cage *A*) females tended to spend more time in the middle third than in other sections of the cage, although the time spent among the three areas of the cage did not vary statistically (one-way repeated measures ANOVA; Control 1, empty cage: $F_{2,18} = 1.13$, $P = 0.34$; Control 2, female stimulus: $F_{2,4} = 0.10$, $P = 0.90$) (Fig. 2). Females thus did not show a propensity for staying close to the adjacent cage when it was either empty or housed a novel female. By contrast, in both manipulations, females presented with a stimulus novel male showed considerable and significant interest for that male. Time spent

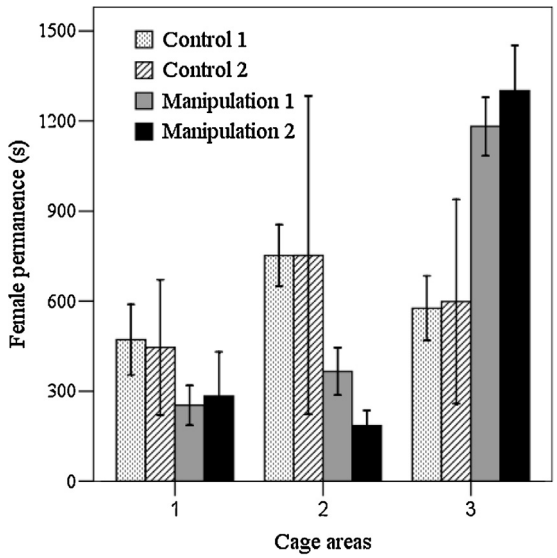

Fig. 2. Female permanence (mean ± standard error) in different areas of the cage where area (3) is closest to the stimulus cage and area (1) is farthest. The stimulus cage varied from being empty (Control 1), to containing a stimulus female (Control 2), or a stimulus novel male (Manipulations 1 and 2). The paired males could only observe the behaviour of the female in Manipulation 1, while in Manipulation 2 they observed the female as well as the novel male. A significant difference was observed for area 3 in Manipulations 1 and 2 in comparison to the other cage areas.

in different areas of the cage varied significantly (Manipulation 1, $F_{1.67,31.80} = 25.48$, $P < 0.0001$; Manipulation 2, $F_{1.20,10.85} = 16.38$, $P = 0.001$), with females spending more time next to the stimulus male (Manipulation 1, Paired Student t tests, both $P < 0.0001$, significant after Bonferroni correction; Manipulation 2, Paired Student t tests, both $P < 0.007$, significant after Bonferroni correction; Fig. 2). Observed behaviour of females in Cage *A* towards novel males included short vocalizations, hopping close to the barrier, attempts to enter Cage *B*, and copulation solicitation (females vibrate their wings and raise their tails, exposing their cloacae).

Female response to novel males generally did not vary as a function of trait properties of those males (body condition index, $r = -0.91$, $N = 11$, $P = 0.78$; plumage reflectance, PC1: $r = 0.23$, $N = 11$, $P = 0.48$; and PC2: $r = 0.33$, $N = 11$, $P = 0.31$). We did, however, observe a nearly significant positive correlation between female response and novel male nuptial plumage coverage ($r_s = 0.58$, $N = 11$, $P = 0.06$). Furthermore, female response to novel males did not vary as a function of relative trait differences between their social mate and the novel male (body condition index: $r = 0.20$, $N = 30$, $P = 0.28$; nuptial plumage coverage: $r_s = -0.24$, $N = 30$, $P = 0.20$; reflectance PC1: $r = -0.06$, $N = 30$, $P = 0.75$; and reflectance PC2: $r = -0.13$, $N = 30$, $P = 0.50$).

3.2. Behaviour of reunited social pairs

3.2.1. Males

When paired males observed the behaviour of their females without visualizing the novel male (Manipulation 1), they exhibited low aggression (0.20 ± 0.16 pecks or chases) towards the female after the pair was reunited. The paired male's resting time during Manipulation 1 interactions did not differ from that during Control 1 interactions ($t_{18} = 0.93$, $P = 0.37$). Also, after Manipulation 1 we did not observe forced copulation attempts by paired males. Thus, paired males either did not recognize changes in paired female behaviour resulting from their exposure to novel males, or else did not react to any changes that were in fact observed.

There was no difference in aggression rate of paired males towards females between the Manipulation 1 and Manipulation 2 treatments (0.16 ± 0.13 versus 0.04 ± 0.04; $U = 45.00$, $N_1 = N_2 = 10$, $P = 0.54$). Moreover, males' resting time did not differ between Manipulations 1 and 2 ($t_{18} = 1.288$, $P = 0.214$), indicating that males were not more restless after observing the female interacting with the novel male. Additionally, there were no forced copulation attempts by paired males.

3.2.2. Females

Across the entire sample, females exposed to novel males (Manipulations 1 and 2) tended to show more aggression towards paired males than did females from the Control 1 treatment (1.70 ± 3.38 [range 0–35] versus 0.28 ± 0.89 [range 0–12] pecks and chases, $U = 100.50$, $N_1 = 30$; $N_2 = 10$, $P = 0.061$). We observed the same trend in terms of the proportion of females in each treatment that responded aggressively: in the Control 1 treatment, only one of ten females was aggressive to her social male, whereas for Manipulations 1 and 2, aggressive behaviour was observed for thirteen of thirty females (43%) (Fisher's Exact test, $P = 0.069$). Females also showed a trend to spend less time close to their partners after being exposed to a novel male (343.23 ± 47.58 s) when compared to females exposed to an empty cage (616.76 ± 130.40 s), although again the difference was not quite statistically significant ($U = 91.00$, $N_1 = 30$, $N_2 = 10$, $P = 0.065$).

Female behaviour directed towards social males did not differ between the two Manipulation treatments (aggression rate: $U = 79.00$, $N_1 = 20$; $N_2 = 10$, $P = 0.285$; time spent close to the partner: $U = 96.00$, $N_1 = 20$; $N_2 = 10$, $P = 0.86$). Females' aggression towards paired males in the two manipulation treatments was not predicted by differences in the social and novel male phenotypes (difference between attacked and non-attacked males, body condition: $t_{28} = 0.39$, $P = 0.70$; nuptial plumage, $U = 79.00$, $N_1 = 10$, $N_2 = 20$, $P = 0.36$; reflectance PC1: $t_{28} = 1.35$, $P = 0.19$ and reflectance PC2: $t_{28} = 0.31$, $P = 0.76$).

3.3. Hormonal analyses

T levels did not differ significantly between males in Manipulation 1 and 2 (4.24 ± 0.16 ng/ml versus 4.07 ± 0.11 ng/ml; $t_{14} = 0.85$, $P = 0.41$). There were also no significant differences in CORT levels (Manipulation 1 males: 3.99 ± 0.93 ng/ml and Manipulation 2 males: 3.53 ± 0.83 ng/ml; $t_{12} = 0.37$, $P = 0.72$). Finally, we detected no relation between the time females spent near the novel males and the physiological response of the paired males (T levels: $r = -0.18$, $N = 16$, $P = 0.50$; CORT levels: $r = -0.24$, $N = 14$, $P = 0.46$).

4. Discussion

Our main results were threefold. First, we found that females who had been paired with males for a lengthy period, equivalent to an entire breeding season (5 months), showed substantial interest for novel males. Females tended to show particular interest in males with a greater coverage of nuptial plumage, which suggests a biological significance given the small sample size during the experiments. However, overall, the interest of the females was not linked to the phenotypic attributes of the novel male or even with differences between the paired male and the novel one. Thus, our results suggest that female preferences are influenced more by the novelty than by the possible quality, both relative and absolute, of novel males' attributes.

Different studies have indicated that the mechanisms of female choice vary broadly, depending on the species, ecological context and ornamental attributes examined. Female rock sparrows (*Petronia petronia*), for instance, are attracted and pay attention to the white tail markings of males, at least during mate choice experiments in captivity (Griggio et al., 2011). Bearded reedling (*Panurus biarmicus*) females, on the other hand, are interested in

novel males independent of their partner' attractiveness (Hoi and Griggio, 2012). In our study, female grassquits appeared to be indiscriminate in the selection of a second male, and this result may be crucial to understand the high level of EPP found in the species (Carvalho et al., 2006). While we do not exclude the possibility of female preference based on traits not investigated in this study, it is possible that females may favour novel males whenever an opportunity presents itself, as a way of enhancing genetic variability of their broods or for other reasons, such as accessing other male territories. Also, perhaps the females we observed were still evaluating males during the 30 min of each experimental trial, and would have only shown a distinction in responses to males based on their quality over a longer time scale.

Our second result of importance concerns the behaviour of paired birds post-trial, when they were reunited in their own cage. Differently from other studies that suggested that coercion may be used by males at risk for loss of paternity (Barash, 1976; Zenone et al., 1979; Johnstone and Keller, 2000; Valera et al., 2003), when paired grassquit males were reunited with their mates they exhibited virtually no aggression. One explanation may be that paired males did not detect the interest of the paired female towards the novel male because they have not evolved the cognitive capacity for interpreting possible behavioural cues. A second explanation is that physical retaliation may not be common in grassquits, which could contribute to the high prevalence of female extra-pair behaviour in the species. But an alternative possibility is that males may have had their visual capacity diminished by viewing through a one-way glass in the laboratory setting. Some studies have shown that diurnal birds are more likely to lose colour vision and detail perception when they are exposed to a low light environment (Lind and Kelber, 2009; Gover et al., 2009; Lisney et al., 2011).

Surprisingly, we found that several females who interacted with novel males were highly aggressive towards their paired males when reunited, whereas control females were not. In addition to aggression, females exposed to novel males tended to stay apart from their mates for more time. To our knowledge, ours is the first study to report female mate aggression associated with the availability of a new mating opportunity. Several studies have shown the importance of females in maintaining the pair bond (Cézilly et al., 2000), others have shown that it is generally the female that opts for divorce (blue tits *Cyanistes caeruleus*: Dhondt and Adriaensen, 1994, but see Valcu and Kempenaers, 2008). In this context, it is possible that the females paired for a lengthy period and without breeding with the first male found in the novel male an alternative breeding option.

The third result of importance concerns the physiological response of social males that saw their mates interacting with a novel male. In addition to not presenting any aggressive response towards the females, paired males also exhibited no responses in the levels of circulating plasma T and CORT to the imminent threat represented by novel males interacting with their mates. Therefore, our data do not support the challenge hypothesis (Wingfield et al., 1990) in terms of male–male competition, at least under these specific laboratory conditions. One explanation for this finding is that the presence of the novel male may simply produce no effect upon the levels of the measured steroid hormones. In other words, elevations in plasma T do not occur in response to socially challenging situations in this species. Alternatively, the proximity of another male to the female, in this species, might not represent a reliable indicator of extra-pair copulation risk, and thus not represent a socially challenging situation. Another possible interpretation, and the one we favour, is that the similar T levels observed in both treatments (with and without visualization of the novel male) is a hormonal response to female presence and hence masks any effects that could have occurred explicitly because of the rival male. In ring doves *Streptopelia risoria* as well as in European starlings *Sturnus vulgaris*, males exposed to females exhibited higher androgen levels than males in the absence of females (Feder et al., 1977; Pinxten et al., 2003), and in the latter species this hormonal response occurred independently of behavioural changes.

5. Conclusions

While numerous studies have measured paternity in wild populations, very little is known about the specific behaviours of males and females that lead up to extra-pair paternity, as these are much more difficult to observe in the field. These findings we report for the blue-black grassquit highlight the importance of female roles in sexual conflict. Blue-black grassquit females showed interest in novel males independent of their phenotypes; and they also showed more aggression to paired males after this exposure. These results suggest that female mate choice strongly emphasizes novelty, at least in the context of sexual pairing, and in the initial time frame in which new males are encountered. These results contribute a new perspective for interpreting the evolution of EPP in this and possibly other species. In short, our results suggest that females of the species determine the preservation or extinction of the pair-bond, depending upon new mating possibilities. The apparently indiscriminate readiness of socially paired females to associate with novel extra-pair males, in addition to a possible lack of behavioural restraints imposed by socially paired males, may together help explain the high levels of EPP in the species (Carvalho et al., 2006; Macedo et al., 2012).

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