

Parasites, mate attractiveness and female feather corticosterone levels in a socially monogamous bird

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Abstract Stress is ubiquitous in the life of animals and a key determinant of their well-being and fitness. By quantifying levels of feather corticosterone in growing feathers (CORTf), we measured integrated stress responses in a monogamous game bird, the red grouse *Lagopus lagopus scoticus*. We investigated the effects of parasites and social mate choice on female CORTf levels during pairing, and tested the hypothesis that females with more parasites and paired with less attractive males have higher CORTf. We experimentally reduced nematode parasite abundance during pairing in females and investigated the effect of treatment on CORTf, while also considering the social mate's phenotype (male comb size, as a proxy of sexual attractiveness). The treatment was effective at con-

trasting parasite loads between control and dosed females, but had no apparent effect on CORTf. In experimental females, reinfection rate after a month positively correlated with CORTf. We found no evidence of assortative mating based on size, condition or ornament size, but females paired with more attractive males (displaying bigger combs) had lower CORTf during pairing. Females for which parasite load was reduced had lower CORTf than control females at all levels of male attractiveness. Social mate choice therefore appears to be an important determinant of female integrated stress responses, which may in turn modulate reinfection rate and parasitism risk. An influence of male attractiveness on female stress may be part of an adaptive response allowing females to adjust reproductive investment to their achieved social mate choice.

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Significance statement

Mate choice is often constrained by the availability of potential mates and not all females socially pair with their preferred partner. We show here that in females of a socially monogamous bird, corticosterone levels in feathers (indicative of integrated stress responses) increase with parasite infection levels but decrease with the attractiveness of the social mate. These results contribute to a better understanding of how females may adjust their reproductive investment according to their achieved mate choice.

Introduction

Many birds form socially monogamous pair bonds in which both mates jointly work towards raising offspring. Mate choice is rarely random: during pairing, females rely on male sexual signals (ornaments, displays) that honestly advertise quality to choose the best possible mate (Andersson 1994). One such aspect of mate quality may be a lower parasite load or greater ability to resist parasites that females can indirectly assess from male ornamental displays, whose expression is often tightly linked to parasite levels (Hamilton and Zuk 1982). Pairing with a mate with fewer parasites may have direct benefits, in terms of reduced parasite transmission risk within pairs (Martinez-Padilla et al. 2012), as well as indirect benefits, such as genes that make offspring more attractive or better able to resist parasites (von Schantz et al. 1996; Møller and Alatalo 1999).

Parasitism is energetically demanding because of the increased energetic costs of coping with the infection (Sheldon and Verhulst 1996) and may, as a result, be associated with increased corticosterone levels (a main avian stress hormone) (e.g. Mougeot et al. 2010; Harriman et al. 2014; Sild et al. 2014). Moreover, greater than needed or prolonged production of corticosterone may result in chronic stress and have detrimental effects, such as immunosuppression and reduced parasite resistance (Romero 2004). Corticosterone has been proposed as a link between parasite infections and ornament expression levels (Mougeot et al. 2010). More recently, mate choice was also shown to influence corticosterone levels: females that lose out in the competition for the best available males will socially bond with a less attractive male and may, as a result, have increased corticosterone levels (Griffith et al. 2011).

Recent advances allow quantifying corticosterone levels in feathers, providing a measure of integrated stress responses (Bortolotti et al. 2008). This opened up a new window of opportunity for understanding the role that physiological stress plays in the life of wild birds (Bortolotti et al. 2009b; Fairhurst et al. 2011; Fairhurst et al. 2012; Koren et al. 2012; Carrete et al. 2013; Lendvai et al. 2013; Martinez-Padilla et al. 2013). However, to date, few studies have related measures of integrated stress responses, such as feather corticosterone levels (hereafter CORTf) to parasite abundance, levels of sexual ornamentation or mate quality (e.g. Bortolotti et al. 2009a, b; Mougeot et al. 2010; Lendvai et al. 2013; Fairhurst et al. 2014; Harriman et al. 2014). Even fewer studies have considered corticosterone levels variation in female birds, and how it relates to parasites, ornamentation and mate quality. We tested here the hypothesis that integrated stress responses in a monogamous female bird depend on both parasite abundance and mate attractiveness. We predicted lower CORTf in females with fewer parasites, and paired with more attractive males, and a possible combined effect of parasites and mate attractiveness on female CORTf levels.

We studied the effects of parasites (as potential stressors) and actual social mate choice on integrated stress responses in free-living female red grouse (*Lagopus lagopus scoticus*) during pairing. An effect of parasitism on CORTf has been documented in previous studies (Bortolotti et al. 2009b, Harriman et al. 2014), indicating that this measure is sensitive enough to detect the effects of parasites. In socially monogamous red grouse, both sexes display conspicuous red combs and comb size functions in intra- and inter-sexual selection. This has been shown in lekking species such as black grouse *Tetrao tetrix*, in which males with bigger combs achieve greater mating success (Rintamaki et al. 2000), as well as in monogamous species, such as the rock ptarmigan *Lagopus mutus* (Bart and Earnst 1999) or the red grouse (Redpath et al. 2006; Mougeot et al. 2007, 2009), in which males with bigger combs hold larger territories, are dominant and preferred by females. A main parasite of red grouse is the nematode *Trichostrongylus tenuis*, which has well documented negative impacts on this host (Martinez-Padilla et al. 2014), reducing condition and comb size in both males (Mougeot et al. 2005a, 2006, 2009) and females (Martinez-Padilla et al. 2011). We sampled social pairs on three study sites before laying and reduced *T. tenuis* parasite abundance in some females using an anthelmintic treatment. We tested whether CORTf was lower and comb size greater in dosed females compared to controls. We also investigated whether a higher CORTf was associated with a greater natural parasite reinfection rate (Bortolotti et al. 2009b), considering parasite burden 1 month after dosing with anthelmintic (previously shown to be effective at removing of *T. tenuis* worms) as an index of reinfection rate. We also investigated whether female CORTf varied according to their social mate's phenotype. In captivity, experimental pairing with a non-attractive has been shown to increase plasma CORT levels (Griffith et al. 2011), so we expected that females paired with more attractive males, displaying bigger combs (Bart and Earnst 1999; Redpath et al. 2006), would have lower CORTf.

Methods

Experiment

In spring 2009 (28 February–19 March), we caught 47 females c.1 month before laying by spotlighting and netting them at night on three UK upland estates (moors) located in northern England (Catterick-Moor1 and Geltsdale-Moor2) and Scotland (Invermark-Moor3). Each individual was fitted with a unique frequency radio collar (TW3-necklace radio tags; Biotrack, Dorset, UK) to facilitate relocation and recapture. We randomly assigned females to one of two parasite treatments: treated females ($n=16$) were orally dosed with 1 ml Levamisole hydrochloride (Nilverm Gold), an

anthelmintic effective at removing *T. tenuis* worms from grouse (Mougeot et al. 2005a; Martinez-Padilla et al. 2012); control females ($n=31$) were orally given 1 ml of water. We also captured the males paired with study females, which were also individually marked and randomly assigned to a parasite treatment (control or dosed, as for females) as part of an experiment looking at parasite transmission within pairs (Martinez-Padilla et al. 2012). One month after the treatment (28 March–5 April), we recaptured all 47 females and their mates whenever possible ($n=42$ pairs in total). It was not possible to record data blind because our study involved focal animals in the field.

Upon initial capture and recapture, all grouse were aged from plumage (two categories: young versus old; young grouse i.e. hatched in the previous summer, have primary flight feathers with pointed tips and small brown dots, while older birds have plain black primaries with rounded tips; Hudson 1986). We measured their comb area (max. length \times width, in mm^2) (Mougeot et al. 2005a), tarsus length (mm), and body mass (g). We calculated their condition (mass adjusted for tarsus length) using a scaled-mass index (Peig and Green 2009). Upon initial capture and recapture, we also collected a fresh faecal sample for estimating *T. tenuis* parasite abundance (Seiwright et al. 2004). To do so, we kept females overnight in individual pens before releasing them early the next morning at the capture site. When birds were captured for the first time, we measured and collected faecal samples for only a sub-sample of study birds, while upon recapture, all study birds were measured and sampled.

Upon recapture, we collected a growing rump feather from each female for corticosterone assays (Bortolotti et al. 2009a). We collected rump feathers that were almost fully grown (c. 90 % of a fully grown size), but that were still growing (i.e. that still had their base in the tubular feather sheath when we plucked them). CORTf was indicative of the amount of corticosterone deposited during the growth of these feathers that took place during the days preceding recapture (from field observations, we estimated that they had probably been growing for a c.2 weeks-period before recapture, in any case in-between the initial capture and recapture date).

Parasite abundance estimates

Faecal samples were stored at 5 °C and analyzed within 2 weeks after collection to ensure reliable parasite abundance estimates (Seiwright et al. 2004). For each, a 0.2-g sub-sample was diluted in 5 ml of saline water and thoroughly mixed. A subsample of this solution was placed in a McMaster slide under microscope ($\times 40$ magnification) to count *T. tenuis* eggs. Numbers of worms per grouse were estimated from faecal egg concentrations (Seiwright et al. 2004).

Feather corticosterone assays

We measured CORT levels in growing rump feathers, in order to estimate corticosterone deposition prior to the recapture (i.e. levels during experiment). Feather corticosterone concentrations were measured by radioimmunoassay (RIA) following a methanol-based extraction (Bortolotti et al. 2008, 2009a; Lattin et al. 2011; Lendvai et al. 2013). Feather length (mm) was measured prior to sample preparation. The calamus of the feathers (including the blood quill) was cut and removed, while the remaining part of the feather was pulverized using a homogenizer into a test tube and was used for the assays. The total mass of the cut feather fragments was weighed to the nearest 0.1 mg. We then added 5 mL methanol to the feather fragments, and the solution was placed in a sonicating water bath for 30 min then incubated overnight in a heated shaker (at 50 °C). The methanol was removed from the feather particles by filtration through a syringe filter (PTFE filter with 0.45 μm pore size, VWR). The tube with the remaining feather particles was washed and filtered twice again with additional 2×2.5 mL volumes of methanol. The methanol was then evaporated under a fume hood at room temperature under a current of air. The extraction was reconstituted with PBS buffer used in the RIA. To determine the efficiency of the extraction, we included samples spiked with a low amount (4000 dpm) of radioactive CORT. Mean recovery was 89.8 %, and final concentrations were corrected by the recovery percentage. We used a commercial antiserum, raised in rabbits against CORT-3-(O-carboxymethyl) oxime bovine serum albumin conjugate (Sigma-Aldrich, St. Louis, MO; product number C8784). According to the manufacturer, the antiserum showed 4.5 % cross-reactivity with cortisol and 3.2 % with cortisone (for a full list see Lattin et al. 2011). The reconstituted extracts were incubated overnight at 4 °C with 100 μL of [^3H]CORT (Perkin Elmer; product number, NET399250UC) and antiserum. The total volume of the assay was 0.4 mL. The radioactively labelled CORT had an activity of ca. 14,000 dpm. Bound and free CORT were separated by adding 500 μL dextran-coated charcoal. After centrifugation, the bound fraction was decanted and added to 5 mL of scintillation cocktail (Bio-Safe II, Research Products Intl.) and counted in a liquid scintillation counter (Beckman LS 6500). Intra-assay coefficient of variation was 6.87 %. CORTf was expressed as pg/mm of feather, as recommended in previous works (Bortolotti et al. 2008, 2009a; Jenni-Eiermann et al. 2015)

Statistical analyses

We used R 3.1.1 (R Core Team 2014; <http://www.R-project.org>) for analyzing the data. Dependent variables were fitted to generalized linear models using a negative binomial (parasite abundance) or to general linear models using normal error distribution (log-transformed CORTf, condition, comb area).

We tested for post-treatment differences in female traits using models that included site, female age, female parasite treatment (control vs dosed) and male parasite treatment (control vs dosed).

Initial models included all these variables and their two-way interactions, which were sequentially removed, starting with interactions, following a backward procedure.

We tested whether CORTf influenced parasite abundance upon recapture using generalized linear models that included CORTf, treatment and the interaction CORTf \times treatment as explanatory variables. We standardized male and female phenotypic data (tarsus length, as a proxy of size, condition and comb area) by site (mean of 0 and SD of 1 within each site) and used GLMs to test for assortative mating.

Results

Before treatment, female comb area varied with female age ($F_{1,41} = 15.56$; $P < 0.001$; old females had bigger combs than young ones), but did not differ between sites ($F_{2,41} = 2.52$; $P = 0.092$) or between female parasite treatment groups ($F_{1,41} = 2.06$; $P = 0.158$). Before treatment, estimated parasite abundance differed between study sites ($\chi^2 = 36.44$; $P < 0.001$; greater estimated parasite abundances in moor 1 and 2 than in moor 3), but did not differ between female age classes ($\chi^2 = 1.16$; $P = 0.281$) or between female parasite treatment groups ($\chi^2 = 1.11$; $P = 0.292$). We thus had no evidence that female comb area or estimated parasite abundance differed between control and dosed females prior to manipulation.

After treatment, estimated parasite abundance tended to be greater in old than young females ($\chi^2 = 3.45$; $P = 0.063$), in moor 1 and moor 2 than in moor 3 ($\chi^2 = 6.72$; $P = 0.034$), was lower in dosed than control females in all sites ($\chi^2 = 61.55$; $P < 0.001$, Fig. 1a), but was unaffected by male parasite treatment ($\chi^2 = 0.24$; $P = 0.618$). Variation in female CORTf measured in feathers grown during experiment was not explained by site ($F_{2,38} = 1.23$; $P = 0.302$), female parasite treatment ($F_{1,38} = 0.97$; $P = 0.33$; Fig. 1b), male parasite treatment ($F_{1,38} = 0.392$; $P = 0.535$) or female age ($F_{1,38} = 0.06$; $P = 0.801$). Interestingly, CORTf explained variation in female estimated parasite abundance upon recapture, depending on female treatment (site $F_{1,39} = 24.78$; $P < 0.001$; female age $F_{1,39} = 3.65$; $P = 0.063$; CORTf \times female treatment $F_{1,39} = 5.55$; $P = 0.024$). In treated females, estimated parasite abundance 1 month after treatment positively correlated with CORTf (GLM controlling for site; partial $F_{1,12} = 6.87$; $P = 0.022$; slope \pm SE $+1.468 \pm 0.560$), whereas no significant association was found for control females (partial $F_{1,25} = 2.15$; $P = 0.112$; slope \pm SE -1.725 ± 1.047).

Variation in female condition post-treatment was not explained by site ($F_{2,37} = 0.09$; $P = 0.917$), CORTf ($F_{1,37} = 0.22$; $P = 0.656$), female parasite treatment ($F_{1,37} = 0.03$; $P = 0.870$),

or male parasite treatment ($F_{1,37} = 1.71$; $P = 0.198$), but differ between female age groups ($F_{1,37} = 11.33$; $P = 0.002$): old females were in better condition (means \pm SE: 0.0123 ± 0.0079 , $n = 22$) than young ones (mean \pm SE -0.0116 ± 0.0065 , $n = 25$). Variation in female comb area post-treatment was not explained by site ($F_{2,36} = 2.27$; $P = 0.117$), CORTf ($F_{1,36} = 0.59$; $P = 0.445$), female parasite treatment ($F_{1,36} = 1.14$; $P = 0.292$; Fig. 1c), male parasite treatment ($F_{1,36} = 0.52$; $P = 0.474$) or female condition ($F_{1,36} = 0.91$; $P = 0.347$), but was explained by female age ($F_{1,36} = 5.59$; $P = 0.024$): old females displayed bigger combs (mean \pm SE 194.4 ± 52.6 , $n = 22$) than young ones (mean \pm SE 149.5 ± 24.3 , $n = 25$).

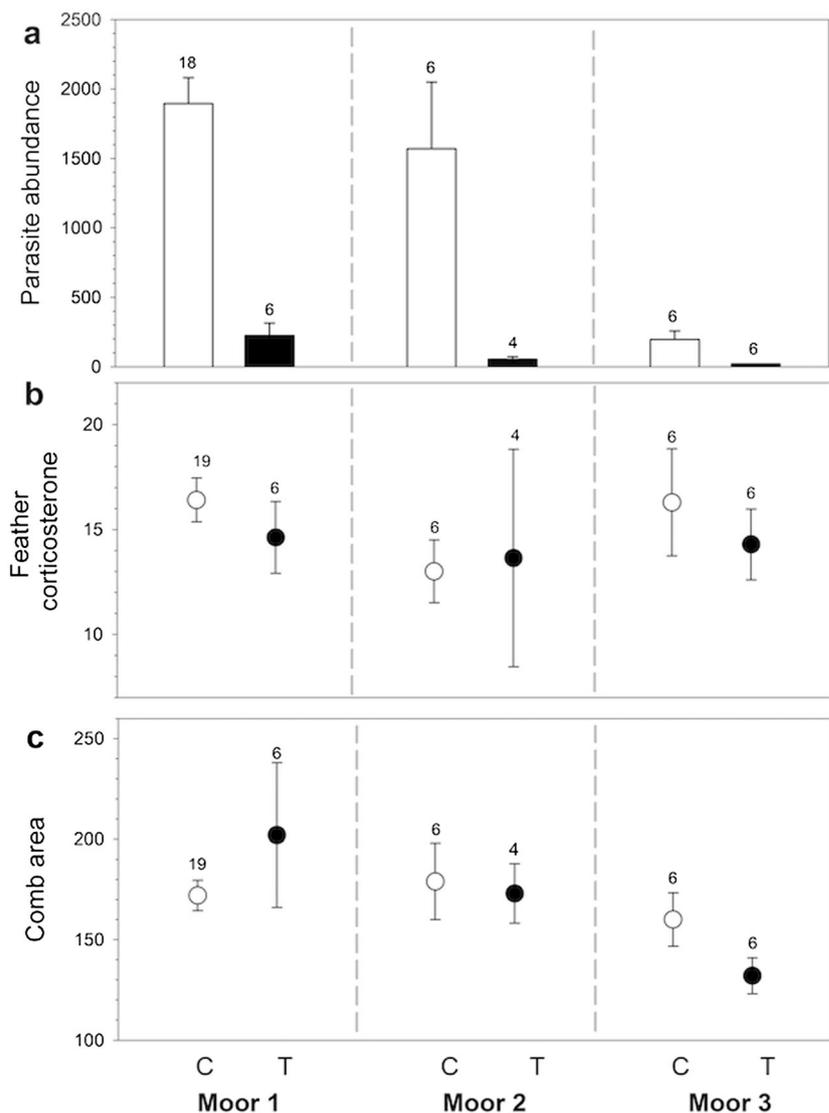
We found no evidence of assortative mating based on size (tarsus length; $F_{1,40} = 0.009$, $P = 0.927$), condition ($F_{1,40} = 0.125$; $P = 0.726$) or comb area ($F_{1,40} = 0.960$; $P = 0.333$). In males, comb area did not differ between sites ($F_{2,39} = 2.33$; $P = 0.111$), age classes ($F_{1,39} = 1.05$; $P = 0.312$) or male parasite treatments ($F_{1,39} = 0.14$; $P = 0.712$). When considering male phenotype, we found that female CORTf was lower in females paired with young than old males ($F_{1,40} = 5.15$; $P = 0.029$; LS means of 12.9 and 15.8 pg/mm, respectively), negatively correlated with male comb area ($F_{1,40} = 10.41$; $P < 0.01$; slope \pm SE -0.045 ± 0.013 ; Fig. 2) and was lower in dosed than in control females ($F_{1,40} = 4.55$; $P = 0.039$; LS means of 13.0 and 15.6 pg/mm, respectively; Fig. 2), with no significant effect of site, female age, female condition or male parasite treatment (all $P > 0.30$).

Discussion

Counts of eggs in faecal samples suggested that treatment was effective at reducing *T. tenuis* nematode parasite abundance in treated female red grouse, as found in previous studies using a similar treatment on red grouse (e.g. Mougeot et al. 2005a, 2007; Martinez-Padilla et al. 2011, 2012). In the studied social pairs, we found no evidence of assortative mating based on body size, condition or comb size, but we found that CORTf was lower in females mated with more attractive males (displaying bigger combs). We also found that when male attractiveness was taken into account, CORTf was also lower in females previously dosed to reduce nematode parasite abundance.

During pairing, social interactions are likely important, as male red grouse actively defend their territory and females may change mate (Redpath et al. 2006; Martinez-Padilla et al. 2012). In red grouse and closely related species, males with bigger combs are more attractive, preferred by females and more often bigamous (Bart and Earnst 1999; Redpath et al. 2006). They are also more aggressive, dominant and hold larger territories (Mougeot et al. 2005a). We have found here that females socially mated to those males had lower CORTf. This suggests that the social pair bond and achieved mate

Fig. 1 Effects of female parasite treatment on nematode parasite abundance, feather corticosterone levels and comb area. Mean \pm SE estimated parasite abundance (number of *T. tenuis* worms per female) (a); feather corticosterone levels (pg/mm) (b); female comb area (mm²) (c) after treatment in control (white bars/dots) and dosed females (black bars/dots) in each of the three study sites (moors 1–3). Numbers above error bars = sample sizes (number of females)



choice influenced corticosterone levels in females (Griffith et al. 2011) or that females with lower CORTf were more successful in acquiring more attractive males. Less ornamented males may provide lower quality territories and less protection from harassment by other males, which could result in increased physiological stress. If so, then we would expect females paired with less ornamented males to be in poorer condition, and a poorer condition to be associated with increased CORTf. Our correlative data suggest that this may not be the case in our study species, as we found no association between female condition and male comb area, or between female condition and CORTf. After forming a social pair bond, females still have scope for optimizing fitness through extra-pair copulations with better quality males or for adjusting their investment in reproduction with respect to the quality of the acquired social partner (Griffith et al. 2011). Defeats following competitive interactions with other females for accessing better mates may result in increased corticosterone levels. Recent

experiments also suggest that corticosterone level variation according to mate attractiveness may be part of an adaptive response to the achieved social mate choice (Griffith et al. 2011), and a mechanism allowing females to adjust accordingly their reproductive investment and optimize fitness (Pryke et al. 2011; Alonso-Alvarez et al. 2012)

Maintaining elevated corticosterone levels over time may have deleterious effects, such as immunosuppression and reduced parasite resistance (Romero 2004). In females initially treated with anthelmintic, we found that reinfection rate 1 month after dosing was greater in sites where parasites were more abundant (moors 1 and 2; Fig. 1a) and where natural exposure to infective larvae was likely greater (Martinez-Padilla et al. 2014). We also found that reinfection rate also positively correlated with CORTf, possibly because the recently picked-up parasites increased CORTf. Parasite infections are energetically costly (Sheldon and Verhulst 1996), and this may result in elevated CORTf (e.g. Harriman et al.

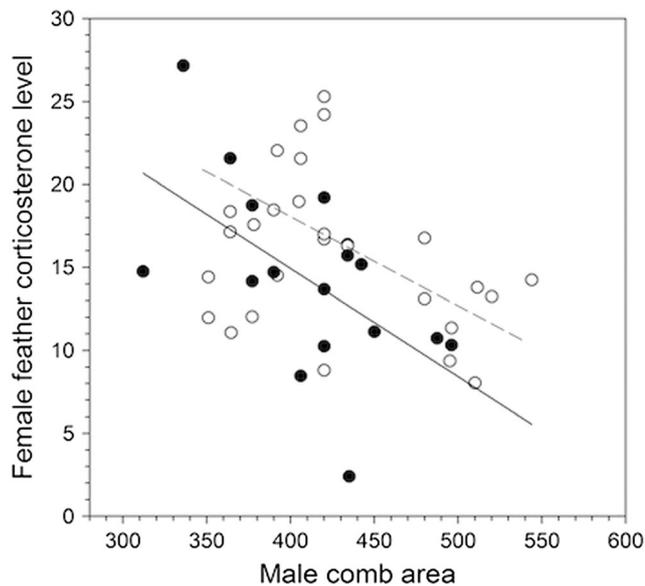


Fig. 2 Female feather corticosterone levels and male attractiveness. Relationships between female feather corticosterone levels (pg/mm) and their social mate's comb area (mm²) in control (white dots) and dosed females (reduced *T. tenuis* parasite abundance; black dots)

2014; Sild et al. 2014) (although estimated parasite abundances may possibly have been too low to have had such an effect). Alternatively, higher corticosterone levels may have induced a greater susceptibility to infective larvae (Mougeot et al. 2005b) and a greater reinfection rate, as also shown in male red grouse following a standardized challenge with infective larvae (Bortolotti et al. 2009b). Previous experiments showed that parasite transmission likely occurs within red grouse social pairs (Martinez-Padilla et al. 2012). Females paired with more attractive males, or with young males that have fewer parasites than old males (Mougeot et al. 2005a; Martinez-Padilla et al. 2014), may thus benefit from a reduced exposure (Martinez-Padilla et al. 2012), reduced corticosterone levels and susceptibility, and therefore reduced overall parasitism risk.

Interestingly, the effects of parasite purging on female CORTf seemed to be subtle and context-dependent, and only became apparent when social mate's attractiveness was taken into account, providing evidence that parasites, under certain circumstances, influence female stress responses (increased CORTf). Social mate choice, which appeared associated with female corticosterone levels (Griffith et al. 2011), may therefore modulate the impacts that natural stressors, such as parasites, have on corticosterone levels. Further research on the mechanisms and functions of physiological stress is needed in free-living animals, and measures of integrated stress responses, like CORTf measurements (Bortolotti et al. 2009b; Fairhurst et al. 2012, 2014; Lendvai et al. 2013) will greatly contribute to this goal. Future experiments should determine whether the association between female corticosterone levels and male ornamentation is mediated via the quality of

resources provided by males to breeding females or is a female adjustment to its achieved mate choice.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures on animals were carried out under a UK Home Office license (PPL 60/3824)

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