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SHORT COMMUNICATION

Prolactin mediates behavioural rejection responses to avian brood parasitism

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ABSTRACT

Adaptations resulting from co-evolutionary interactions between avian brood parasites and their hosts have been extensively studied, yet the physiological mechanisms underlying antiparasitic host defences remain little known. Prolactin, one of the main hormones involved in the regulation of avian parental behaviour, might play a key role in the orchestration of the host responses to avian brood parasitism. Given the positive association between prolactin and parental behaviour during incubation, decreasing prolactin levels are expected to facilitate egg-rejection decisions. We tested this prediction by implanting Eurasian blackbird (*Turdus merula*) females with an inhibitor of prolactin secretion, bromocriptine mesylate, to experimentally decrease their plasma prolactin levels. Bromocriptine mesylate-implanted individuals ejected mimetic model eggs at higher rates, and showed shorter latency to egg ejection, than placebo-treated birds. To our knowledge, this is the first experimental evidence that behavioural host defences against avian brood parasitism are mediated by prolactin.

KEY WORDS: Egg-rejection decisions, Hormones, Host species, Parental behaviour

INTRODUCTION

Obligate avian brood parasites lay their eggs in the nest of other bird species (hosts), thereby exploiting the parental care that host species provide to their offspring (Roldán and Soler, 2011). This reproductive strategy imposes severe fitness costs on hosts as the newly hatched parasitic nestling often eliminates or outcompetes the host's offspring (Feeney et al., 2014; Soler, 2014). In response, many hosts have evolved a wide range of behavioural, morphological and life-history adaptations to counteract brood parasitism at all stages of the breeding cycle (Feeney et al., 2014; Soler, 2014, 2017). While the focus has been on the ecological, cognitive and conditional aspects of host resistance to avian brood parasitism (Davies, 2000; Soler, 2017; Stokke et al., 2008), the endocrine mechanisms underlying antiparasitic behaviour have received comparatively little attention despite their potential role in the evolution and expression of host defences (Abolins-Abols and Hauber, 2018; Avilés, 2018).

The recognition and subsequent rejection of brood parasitic eggs is the most widespread and effective antiparasitic host defence (Davies and Brooke, 1989; Feeney et al., 2014; Soler, 2014). Individual thresholds for egg rejection are determined by the degree of phenotypic dissimilarity between the host eggs' appearance and the parasitic egg, yet some hosts can flexibly adjust their egg-rejection decisions according to previous experience or the perceived risk of brood parasitism (Ruiz-Raya and Soler, 2020). Mechanistically, egg rejection involves the selective disruption of typical parental behaviours to actively respond to a specific stimulus from their own clutch (the parasitic egg). The host response to parasitic eggs, including flexible adjustments in rejection thresholds, has been hypothesized to be mediated by hormone pathways linked to the regulation of main parental decisions (Abolins-Abols and Hauber, 2018; Ruiz-Raya and Soler, 2020). Recent evidence suggests that physiological and behavioural host responses to foreign eggs could be regulated by the neuroendocrine pathways underlying stress physiology (corticosterone: Abolins-Abols and Hauber, 2020; Ruiz-Raya et al., 2018). Nevertheless, whether egg-rejection decisions are mediated by the endocrine pathways associated with the expression of avian parental care is unclear.

Prolactin, a pituitary hormone classically known as the 'parental hormone' (Buntin, 1996; Sockman et al., 2006), is the primary candidate for orchestrating anti-parasitic defences associated with parental decisions (Abolins-Abols and Hauber, 2018). In birds, plasma prolactin levels increase markedly from incubation to post-hatching periods (Angelier et al., 2016; Buntin, 1996; Smiley, 2019; Sockman et al., 2006). Prolactin plays a key role in the regulation of major aspects of avian parental care: circulating prolactin is positively related to the initiation and maintenance of associative parental behaviours such as egg incubation or post-hatching parental care (Angelier and Chastel, 2009; Angelier et al., 2016; Smiley, 2019). Avian brood parasitism is known to affect the stress-induced prolactin response in adult hosts (Ruiz-Raya et al., 2018); thus, it is plausible that this hormone may be involved in the regulation of antiparasitic behaviours (Abolins-Abols and Hauber, 2018; Ruiz-Raya and Soler, 2020). Given the positive association between plasma prolactin levels and the expression of avian parental behaviours (Angelier et al., 2016; Smiley, 2019), it can be hypothesized that lowering circulating prolactin can affect the host's propensity to reject brood parasitic eggs (Abolins-Abols and Hauber, 2018). Here, we tested this hypothesis for the first time by implanting host females with bromocriptine mesylate (BRC) pellets, a D2 dopamine receptor agonist, to experimentally decrease their prolactin levels. Then, we assessed their response to experimental parasitism compared with placebo-implanted individuals in order to determine whether changes in prolactin profiles affect egg-rejection decisions. Decreasing prolactin levels was expected to result in more restrictive thresholds for egg acceptance, thereby facilitating rejection decisions (Abolins-Abols and Hauber, 2018; Ruiz-Raya and Soler, 2020). We predicted that BRC-implanted females would eject parasitic model eggs at higher rates compared with placebo-implanted individuals.

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MATERIALS AND METHODS

Study system

This study was conducted on a Eurasian blackbird (*Turdus merula* Linnaeus 1758) population located in the Valley of Lecrín, Southern Spain, from late March to early June 2018. The Eurasian blackbird (hereafter blackbird) is an occasional host to common cuckoo (*Cuculus canorus*), frequently used in egg-rejection studies (e.g. Grim et al., 2011; Roncalli et al., 2019; Ruiz-Raya et al., 2015; Samas et al., 2011; Soler et al., 2015, 2017). Blackbirds have strong ejection abilities and typically remove foreign eggs by grasping them with their bill (see references above). Egg ejection is mainly conducted by females (the incubating sex in this species), whereas male blackbirds do not show the ability to recognize mimetic eggs (Ruiz-Raya et al., 2019). Desertion rates in experimentally parasitized blackbird clutches are similar to those found in unparasitized nests, so nest desertion is not considered a specific response to brood parasitism in this species (Soler et al., 2015).

Ethics

This study followed all relevant Spanish national (Decreto 105/2011, 19 dee Abril) and regional guidelines. No individual exhibited long-term negative effects as a consequence of our treatment.

Hormone manipulation

From the beginning of the breeding season, we actively searched for blackbird nests and randomly selected 36 females for hormone manipulation. Replacement clutches were not used in this study. Individuals were captured just after clutch completion (mean±s.e.m., 2.3±0.19 days) by using a mist net placed near the nest (06:00–08:00 h; 1–5 m from the focal nest). Blood samples were collected from the brachial vein with a 25-gauge needle and 80 µl heparinized a tubes within 3 min of capture (mean±s.d. 2.24±0.27 min). Afterwards, we randomly assigned birds to one of two experimental groups. In the BRC group, individuals were implanted with bromocriptine mesylate (BRC; n=18) time-release biodegradable pellets (according to the characteristics of our study species: C-231, 0.5 mg, 10 day release, 3.1 mm diameter; Innovative Research of America) following a widely used methodology to lower circulating prolactin in avian species (e.g. Angelier et al., 2006; Cottin et al., 2014; Smiley and Adkins-Regan, 2018; Thierry et al., 2013). In the control group, females were implanted with placebo biodegradable pellets (n=18; C-111, 0.5 mg, 10 day release, 3.1 mm diameter; Innovative Research of America). Time-release dosage pellets have been frequently used to modify hormonal levels (e.g. prolactin, corticosterone) in ecophysiological studies, showing no negative long-term effect for bird welfare (e.g. Cottin et al., 2014; Müller et al., 2009; Thierry et al., 2013). Even so, in order to account for unintended side effects of BRC treatment on avian feeding behaviour (Buntin, 1989), females were weighed to the nearest 1 g before and after the hormonal treatment to assess changes in their body mass (Smiley and Adkins-Regan, 2018). Additionally, we assessed whether our manipulation impacted the probability of nest abandonment. Prolactin levels were not affected by bleeding time either in BRC- or in placebo-implanted females (both cases P>0.55; Fig. S1a).

BRC- and placebo-implanted individuals underwent identical procedures. Pellets were implanted subcutaneously in the female's back through a small incision (5 mm) that was subsequently closed without suture by using surgical tissue adhesive (Vetbond, 3M). All females were marked with a combination of coloured rings to verify their identity and released 10 min after implantation. We visited the focal nests 2 h after female release to assess whether clutches were warm, which confirmed that all females returned to their nests to resume incubation within the next 2 h. Female identity was confirmed by filming female behaviour at the nest (the next day; see below). To test the effectiveness of the BRC pellets, females were recaptured 6 days after implantation (06:00–08:00 h) and a second blood sample was collected from the opposite wing (mean±s.d. 2.42±0.24 min; n=9), which allowed us to assess the females' prolactin levels at the end of the experimental parasitism trials. Recapture procedures with similar sample sizes have been successfully used to verify hormone implants/injection effectiveness in previous studies (Abolins-Abols and Hauber, 2020; Goutte et al., 2010; Ouyang et al., 2013), so we minimized the number of re-sampled females to reduce the stress linked to recapture and handling for breeding pairs. The visual examination of recaptured individuals revealed that incisions were successfully closed in all cases. Blood samples were kept cold for up

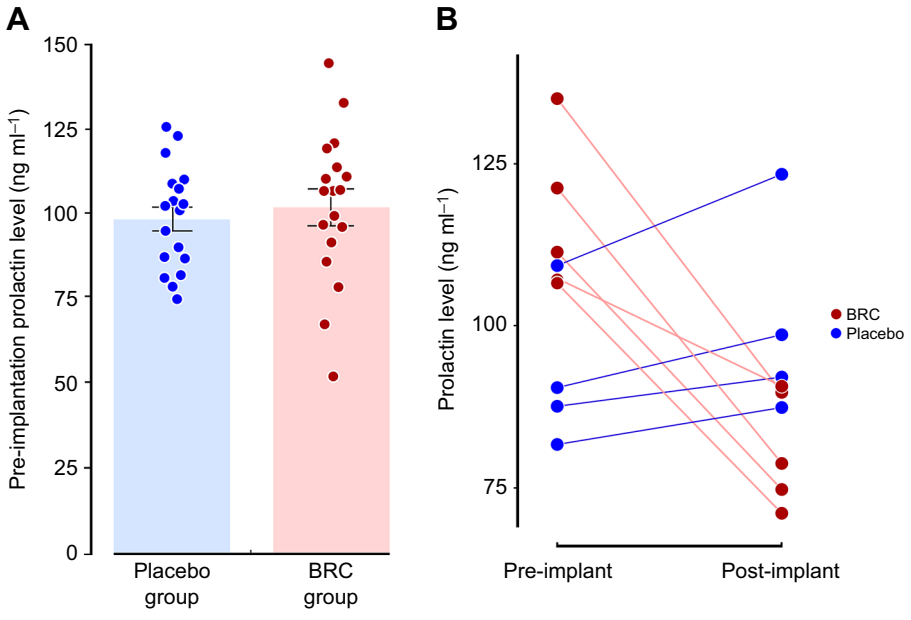

Fig. 1. Effect of bromocriptine mesylate (BRC) implants on prolactin levels in female blackbirds. (A) Pre-treatment prolactin levels (i.e. before implantation) in females from the placebo (n=18) and BRC-implanted (n=18) groups. Values are means±s.e.m. (B) Effect of BRC implants (10 day release pellets) on females' prolactin levels 6 days after implantation (recaptured individuals; n=9). BRC-implanted females showed a significant reduction in circulating prolactin post-treatment compared with placebo-implanted individuals.

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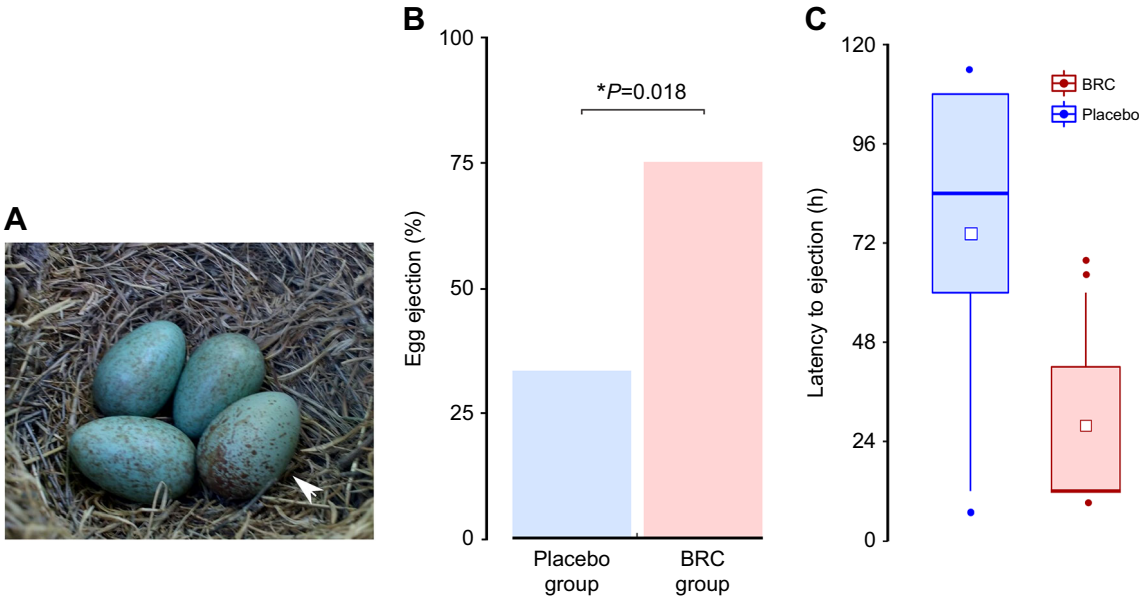

Fig. 2. Egg-ejection behaviour. (A) A blackbird clutch experimentally parasitized with a mimetic model egg (white arrow). (B) Probability of egg ejection in both BRC- (red; *n*=16) and placebo-implanted females (blue; *n*=15). (C) Differences in the latency to egg ejection between BRC- and placebo-implanted females. Boxplots show the median (bold line), mean (white box), and 25th and 75th percentiles (coloured boxes), with whiskers denoting the 5th and 95th percentiles.

to 6 h until centrifugation. Afterwards, plasma was extracted and stored at −20°C. Pre-treatment prolactin did not significantly change over the breeding season for both BRC- and placebo-treated females (both cases $P>0.57$; Fig. S1b).

Brood parasitism experiments

Nests were parasitized with mimetic eggs 24 h after pellet implantation. As model eggs, we used natural blackbird eggs collected from deserted clutches, which were painted to simulate interspecific parasitism (Fig. 2A; see Soler et al., 2015, for additional details). This type of mimetic model egg elicits intermediate ejection rates in blackbirds (26.8–50%; Roncalli et al., 2019; Soler et al., 2015), which allowed us to assess whether our BRC manipulation either enhanced or reduced egg ejection. A video camera was hidden in a nearby tree (3–4 m away) to film the female behaviour for 2 h after the experimental parasitism. Recordings were used to verify the identity of focal females and assess their incubation patterns (the proportion of time spent incubating per hour). All nests were checked every 24 h to determine egg ejection. Experimental eggs were considered as accepted if they remained in active nests for 5 days (Roncalli et al., 2019; Ruiz-Raya et al., 2015, 2016; Soler et al., 2017).

Hormone assays

Hormonal analyses were performed at the Centre d'Etudes Biologiques de Chizé (CEBC, France). Prolactin (PRL) plasma concentration was determined by a heterologous radioimmunoassay (RIA) following the method described by Cherel et al. (1994) and validated for blackbirds (Préault et al., 2005). Briefly, the assay was carried out by a double-antibody (Ab) method with a rabbit anti-chicken PRL antiserum (IFP-151) and a highly purified chicken-PRL preparation (AFP-4444B; both supplied by Dr A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). PRL was measured directly in plasma which was incubated overnight at 4°C with 8000 CPM of ^{125}I-chicken-PRL and the polyclonal antiserum. The bound fraction (Ab-prolactin or Ab-labelled prolactin) was separated from the free fraction (prolactin and labelled prolactin) by immunoprecipitation with a second Ab against PRL antiserum (sheep anti-rabbit Ab). The activity of the bound fraction was counted in a Wizard Gamma counter 2470 (Perkin Elmer). All samples were run in the same assay, in duplicate, with 25 µl of plasma for each duplicate and a limit of detection of 0.43 ng ml^{-1}. Repeatability estimates were calculated via parametric bootstrapping by using the *rptR* R package (Stoffel et al., 2017). Hormone assays were highly repeatable (r=0.88; 97.5% CI 0.79, 0.93; $P<0.0001$; intra-assay CV 10.3%).

Statistical analysis

We used linear models (LMs) to explore whether the experimental groups (BRC versus placebo) differed in their pre-treatment prolactin levels. LMs were also used to assess potential changes in plasma prolactin through the breeding season in both experimental groups, as well as the effect of bleeding time on plasma prolactin concentration (n=36). The effects of our BRC manipulation on both circulating prolactin levels and body mass in re-captured females (n=9) were analysed by fitting linear mixed-effect models (LMMs; *nlme* R package v.3.1-117; http://CRAN.R project.org/package=nlme>). As fixed factors, mixed models included implant treatment (BRC or placebo), sample day (the day on which the focal females were captured; day 1=1 April), and the two-way interaction between these terms, while female ID was included as random factor. We used generalized linear models (GLMs) with binomial error to explore the effect of our implant treatment (BRC or placebo) on both the probability of nest desertion (yes/no, n=36) and the egg-ejection response to experimental brood parasitism (yes/no) in those nests that completed the 5 day experiment (i.e. active nests, n=31). We also used binomial GLMs to examine whether our implant treatment affected the incubation patterns of female blackbirds (i.e. proportion of time spent incubating; binomial distribution; n=31). Finally, we used LMs to assess whether our BRC treatment affected the blackbirds' latency to egg ejection (Box–Cox transformed; n=31). *Post hoc* comparisons were performed by using the *lsmeans* R package (Lenth, 2016). Assumptions for normality of residuals and

homogeneity of variances were verified, when necessary, by the inspection of diagnostic plots for residuals. All analysis and graphs were performed using R version 3.6.1 (http://www.R-project.org/).

RESULTS AND DISCUSSION

Experimental groups did not differ in their pre-implantation prolactin levels (LM, $F_{1,34}$=0.28, P=0.60; Fig. 1A). The BRC treatment significantly impacted blackbirds' prolactin levels (LMM; implant treatment × sample day: $F_{1,7}$=52.17, P<0.001; Fig. 1B). Specifically, plasma prolactin concentrations decreased in BRC-implanted individuals (Tukey's *post hoc* test, P<0.001), while placebo-treated birds showed similar hormone levels after implantation (Tukey's *post hoc* test, P=0.34). Our results therefore confirm that BRC treatment leads to an effective decrease in plasma prolactin levels in Eurasian blackbirds, as previously shown in other bird species (e.g. Angelier et al., 2006; Cottin et al., 2014; Smiley and Adkins-Regan, 2018; Thierry et al., 2013), even though subsampling might potentially have overstated the effects of our BRC manipulation. It is noteworthy that the suppression of prolactin secretion through BRC can impact metabolic homeostasis (e.g. glucose and lipid metabolism: Ben-Jonathan et al., 2006) and food intake in mammals (Bonomo et al., 2005). In our study, pellet implantation did not affect blackbirds' body mass (LMM; sample day: $F_{1,7}$=1.98, P=0.20), regardless of the hormone treatment (LMM; implant treatment×sample day: $F_{1,7}$=1.02, P=0.35; Fig. S2a). These findings support previous studies showing absence of unintended side effects in BRC-treated birds (Smiley and Adkins-Regan, 2018).

BRC-implanted hosts ejected mimetic model eggs at significantly higher rates than placebo-treated individuals (GLM, χ^2=5.59, d.f.=1.29, P=0.018; Fig. 2B), supporting our prediction that the reduction of plasma prolactin levels would facilitate and promote egg-rejection decisions. Egg-ejection rates shown by placebo-implanted blackbirds were similar to those found in previous studies where no hormonal manipulation was performed (28.6%; Soler et al., 2015). Elevated prolactin levels are needed for the maintenance of parental behaviour during egg incubation (Angelier and Chastel, 2009). It seems plausible that the suppression of prolactin secretion may reduce incubation effort and attentiveness to eggs, and eventually impact rejection decisions. BRC- and placebo-implanted females did not differ in the time that they spent incubating on the day after implantation (GLM, χ^2=0.14, d.f.=1.29, P=0.71; Fig. S2b), yet we cannot discard changes in incubation patterns over subsequent days. Importantly, latency to ejection was shorter in BRC- than in placebo-implanted females (LM, $F_{1,15}$=12.82, P=0.002; Fig. 2C). To the extent of our knowledge, this is the first empirical evidence that the probability and timing of egg rejection may be mediated by variations in circulating prolactin.

Plasma prolactin levels have been shown to decrease in response to stressors in many bird species (Angelier and Chastel, 2009; Angelier et al., 2016). Thus, individual variation in the egg-rejection behaviour might be associated with differences in prolactin sensitivity to environmental challenges. It is well known that some hosts rely on the combined use of personal (e.g. brood parasite presence) and social information (e.g. alarm calls from neighbouring territories) about the local risk of brood parasitism to plastically adjust their acceptance thresholds according to the current environmental context (Thorogood and Davies, 2016). Meadow pipits (*Anthus pratensis*), for example, require both a parasitic egg in the nest and the presence of a cuckoo female to reach the threshold for egg rejection (Moksnes et al., 1993). One might predict that increased risk of brood parasitism (e.g. witnessing a parasite near the nest) would lead to a drop in prolactin levels and eventually impact egg-rejection decisions, which would explain why the sight of a adult brood parasite at the nest facilitates egg rejection in some hosts (Bartol et al., 2002; Moksnes et al., 1993, 2000). Brood parasitism is known to impact adult hosts' hormone levels during the incubation (Ruiz-Raya et al., 2018), nestling (Antonson et al., 2020) and fledgling stages (Mark and Rubenstein, 2013). Maternal hormone investment in host eggs is correlated with brood parasitism (Hahn et al., 2017) and might determine the host's propensity to reject parasitic eggs (Hauber et al., 2020). However, the extent to which the presence of adult brood parasites affects the hormonal levels of hosts is unknown. Recent evidence has shown that the prolactin stress response is more pronounced in individuals experimentally parasitized with non-mimetic eggs (Ruiz-Raya et al., 2018), which suggests that prolactin responsiveness to brood parasite presence might be higher after perceiving a first cue of brood parasitism (e.g. an odd egg in the nest).

Importantly, prolactin mediation of avian parental care can involve additional neuroendocrine pathways (e.g. corticosterone; Angelier et al., 2016). It is therefore possible that prolactin interacts with other hormones to regulate host responses towards avian brood parasitism. Lowering plasma corticosterone has been shown to increase egg acceptance in American robins (*Turdus migratorius*), a brown-headed cowbird (*Molothrus ater*) host, which is phylogenetically very close to Eurasian blackbirds (Abolins-Abols and Hauber, 2020). Taken together, these results suggest that egg rejection may be under the control of multiple endocrine mechanisms, drawing attention to the need for integrative studies combining prolactin and corticosterone. Long-term exposure to elevated corticosterone levels can affect prolactin secretion in adult birds (e.g. Criscuolo et al., 2005; Spée et al., 2011), although circulating levels of the two hormones are not often correlated (reviewed in Angelier et al., 2013). Thus, corticosterone and prolactin could provide complementary information on the proximate mechanisms meditating egg-rejection decisions.

Decreasing prolactin levels are associated with higher probabilities of nest abandonment and low breeding success in many birds (reviewed in Angelier et al., 2016), although this link is not straightforward in all species (Angelier et al., 2016; Kosztolányi et al., 2012; Wojczulanis-Jakubas et al., 2013). In our study, five out of 36 females (13.8%) deserted their nest, but desertion probabilities did not differ between BRC- and placebo-implanted females (16.7% placebo, 11.1% BRC; GLM, χ^2=0.23, d.f.=1.34, P=0.63). Interestingly, these desertion rates were similar to those previously found in non-hormonally manipulated individuals from the same population (21.4%; Soler et al., 2015). Our results are therefore consistent with previous research showing that prolactin decrease is not a reliable predictor of nest desertion in experimentally parasitized blackbird clutches (Ruiz-Raya et al., 2018). Nest desertion may be under the control of different neuroendocrine pathways, and depend on the energy state of individuals (Angelier et al., 2016; Kosztolányi et al., 2012). It is also worth noting that, while blackbirds do not use nest desertion as an antiparasitic defence (Soler et al., 2015), prolactin could mediate nest-desertion responses to brood parasitism in host species that do use nest desertion as an antiparasitic defence, as may be the case in some brown-headed cowbird hosts (Hosoi and Rothstein, 2000), or small-sized species unable to eject brood parasitic eggs (Davies, 2000).

To sum up, we provide experimental evidence that prolactin can mediate the host response to avian brood parasitism. Future field studies exploring natural variations in prolactin levels, the strength of the prolactin response to stressors, or individual differences in

target tissue sensitivity to hormones (e.g. prolactin receptor density) will be particularly helpful to improve our understanding on the hormonal mechanisms underlying differences in egg-rejection behaviour between and within host populations. Further research is also needed to establish to what extent the relationship between lowering prolactin and hosts' behavioural phenotypes is causal or, instead, it may be the result of interaction with other physiological factors. Finally, research on the endocrine basis of antiparasitic defences needs to be extended to other lines of host defence in which parental decisions are expected to be crucial (e.g. rejection of parasitic nestlings; Grim, 2017).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: F.R.-R., J.D.I.-A., M.S.; Methodology: F.R.-R.; Software: F.R.-R.; Validation: F.R.-R.; Formal analysis: F.R.-R., C.P., O.C.; Investigation: F.R.-R.; Resources: M.S.; Data curation: F.R.-R.; Writing - original draft: F.R.-R.; Writing - review & editing: F.R.-R., J.D.I.-A., C.P., O.C., M.S.; Visualization: F.R.-R.; Funding acquisition: M.S.

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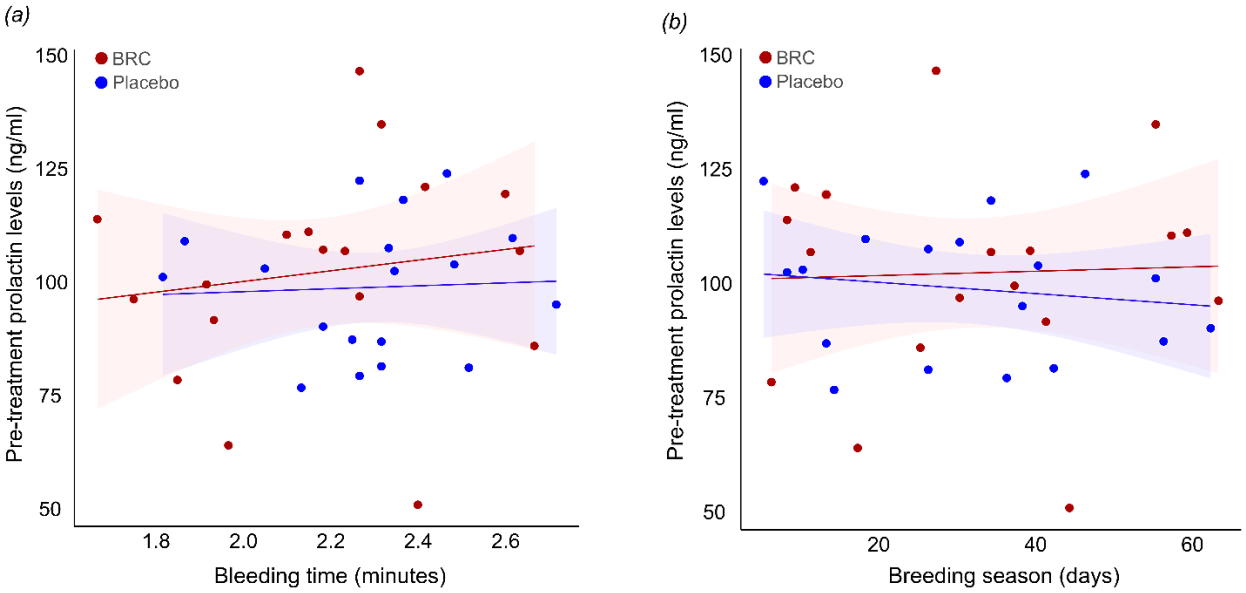

Fig. S1. **(a)** Relationships between bleeding time and pre-treatment prolactin levels. Bleeding time did not affect pre-treatment prolactin levels in BRC (estimate ± se = 11.78 ± 19.29; t = 0.611; p = 0.55) and placebo-implaned females (estimate ± se = 3.27 ± 15.98; t = 0.21; p = 0.84). **(b)** Variations in pre-treatment prolactin levels through the breeding season. There was no seasonal variation in prolactin levels before implantation for both BRC (estimate ± se = 0.05 ± 0.31; t = 0.155; p = 0.88) and placebo-implaned females (estimate ± se = -0.12 ± 0.21; t = -0.58; p = 0.57).

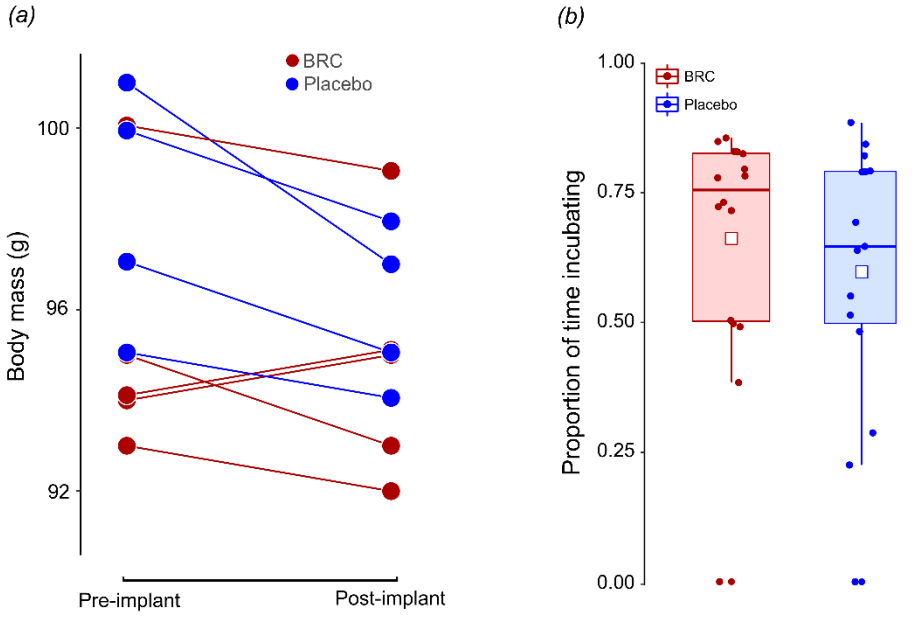

Fig. S2. **(a)** Changes in body mass 6 days after experimental manipulation. **(b)** Proportion of time spent by female blackbirds incubating their eggs. Box plots show the median (continuous line), the mean (white box), and 25th and 75th percentiles (colored boxes), with whiskers denoting the 5th and 95th percentiles.