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Hormonal responses to non-mimetic eggs: is brood parasitism a physiological stressor during incubation?

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Abstract

Many host species have evolved sophisticated defences to mitigate the high fitness costs imposed by brood parasitism. Even though the physiological mechanisms behind such defences can offer important insights into the evolutionary relationship between brood parasites and hosts, they have received little attention so far. Hormones play a critical role in the regulation of bird reproduction, which make them a key element when investigating the physiological effects of brood parasitism on hosts. Here, we experimentally parasitized Eurasian blackbird (*Turdus merula*) nests with non-mimetic eggs to study its impact on the hormonal levels (corticosterone and prolactin) of females during incubation, as well as the magnitude of the response to the standardized stress protocol in parasitized and non-parasitized individuals. Parasitized females had higher baseline corticosterone levels and showed a poorer body condition than non-parasitized birds, while we found no differences for prolactin levels. Both parasitized and non-parasitized females responded to the standardized-stress protocol with a significant increase in corticosterone levels. However, the decrease in prolactin after the standardized stress protocol was significantly more pronounced in parasitized individuals. Our results suggest that the presence of a non-mimetic parasitic egg involves a stressful situation for hosts, negatively affecting the physical state of parasitized females. Unaffected prolactin levels of parasitized individuals could explain the absence of nest desertion found in this species in response to parasitism. Finally, both hormones were not correlated in blackbirds, confirming that their combined study provides valuable pieces of information on the endocrine mechanisms underlying behavioural responses in animals, including hosts of brood parasites.

Significance statement

Physiological mechanisms behind avian brood parasitism remain unclear. In this study, we assessed the effect of experimental parasitism on the hormonal profiles of hosts. We found that the presence of a non-mimetic egg in the nest modified baseline corticosterone levels, but not prolactin levels, of parasitized females and negatively impacted their body condition. Moreover, experimental parasitism affected the prolactin response to stress. These results expand previous information on the endocrine consequences of brood parasitism at other stages of the breeding cycle (nestling and fledgling stage) and might shed light on the hormonal mechanisms that underlie the host response against parasitic eggs.

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Introduction

Interspecific avian brood parasites exploit the parental care that other species provide to their offspring and impose high fitness costs on hosts since the parasitic chick is usually a better competitor for food or evicts all host offspring (Davies 2000; Roldán and Soler 2011; Soler 2014). In response, some host species have evolved defences that enable them to avoid such costs, egg rejection being the most widespread and effective of anti-parasitic behaviours (Davies 2000; Soler 2014). As what occurs with other environmental stressors, brood parasitism induces individuals to modulate their behaviour to optimize their responses. Thus, given the importance of the endocrine pathways on the behavioural adjustments of birds, hormones are excellent candidates to explore the poorly studied physiological mechanisms underlying these anti-parasitic behavioral responses. As far as we know, only three studies have investigated the consequences that brood parasitism has on the endocrine profile of hosts. Ibáñez-Álamo et al. (2012) showed that great spotted cuckoo (*Clamator glandarius*) parasitic chicks induced an elevation of corticosterone (CORT hereafter) levels in the nestlings of its main hosts, the magpie (*Pica pica*), especially during periods of high food requirements. In addition, it has been found that parents parasitized by the Central American striped cuckoo (*Tapera naevia*) showed higher stress-induced CORT levels than non-parasitized parents during the fledgling stage, which involved important fitness costs in subsequent breeding seasons (Mark and Rubenstein 2013). Finally, a recent study including three host species of the brown-headed cowbird (*Molothrus ater*) has highlighted the importance of maternal androgens in the anti-parasitic response of some hosts. In addition, maternal androgens could also play a key role in the brood parasites' response to intra-specific competition in multiply-parasitized nests (Hahn et al. 2017). These studies have provided crucial information to understand the endocrine mechanisms associated with brood parasitism; however, how parasitic eggs impact the host physiology during incubation, where the most important host defence (i.e. egg rejection) has evolved, remains unknown.

From a general perspective, environmental stressors induce the allocation of available resources from reproduction to behaviours enhancing survival (concept of "allostasis") (Wingfield et al. 1998; Wingfield and Sapolsky 2003; Angelier and Chastel 2009). Glucocorticoid hormones have been suggested to play a key role in this context, particularly in birds (Wingfield and Hunt 2002; Angelier and Wingfield 2013). Specifically, CORT is considered as one of the principal mediators in the physiological allostasis: circulating levels of this hormone increase in response to a stressful situation, leading to physiological and behavioural changes that promote individual's survival over other activities (i.e. reproduction) (Wingfield et al. 1998; Breuner et al. 2008). However, when the CORT response to stress has a low adaptive value, it can be down-regulated in order to provide fitness benefits to individuals (Lendvai et al. 2007; Lendvai and Chastel 2008, 2010; Heidinger et al. 2010; Addis et al. 2011; Goutte et al. 2011), making CORT a key tool to understand the individuals' responses to environmental perturbations (Wingfield and Sapolsky 2003; Hau et al. 2010; Wingfield et al. 2011), including brood parasitism (Ibáñez-Álamo et al. 2012; Mark and Rubenstein 2013).

A second hormone that could play a critical role in the brood parasitism context because of its importance regulating parental care in birds is prolactin, a pituitary hormone associated to the expression of avian incubating and brooding behaviours (Buntin 1996). Previous studies on the hosts' physiology have focused mainly on CORT (Ibáñez-Álamo et al. 2012; Mark and Rubenstein 2013; Hahn et al. 2017) while prolactin has been largely overlooked despite its importance in the physiology of avian breeding (Angelier et al. 2016a). It has been found that maintenance of parental behaviour is linked to elevated levels of prolactin whereas low levels are usually related to breeding failure and nest desertion (Sockman et al. 2006; Angelier and Chastel 2009; Ouyang et al. 2011). Prolactin is also affected by environmental stressors in birds and circulating levels of this hormone decrease after exposure to an acute stressor, which can lead to the disruption of current parental care and nest desertion in order to redirect the energy from reproduction to self-maintenance (Angelier et al. 2007; Angelier and Chastel 2009). However, the prolactin response to stress can be down-regulated in order to maintain parental care if current reproduction has an important fitness value (Chastel et al. 2005; Angelier et al. 2007; Angelier and Chastel 2009; Heidinger et al. 2010). The magnitude of the decrease in prolactin levels in response to the standardised stress protocol has therefore been suggested to reflect the individual's parental investment in the current reproduction (Angelier and Chastel 2009).

CORT and prolactin responses to stress have been rarely investigated together despite that they provide complementary information on parental investment in birds (Angelier et al. 2013). Some studies have pointed out an effect of CORT on prolactin secretion (Angelier et al. 2009a; Tartu et al. 2015); however, most studies failed to find a link between these hormones (reviewed in Angelier et al. 2013), suggesting that they could mediate different behavioural responses to acute stressors (Angelier et al. 2013, 2016b; Krause et al. 2015). The combined study of both CORT and prolactin could therefore provide a new perspective in understanding how brood parasitism impacts both the physiology and behaviour of host species.

Here, we experimentally parasitized natural nests of Eurasian blackbirds (*Turdus merula*; blackbird hereafter) with non-mimetic eggs to study the effect of experimental brood parasitism on the hormonal and physical state of hosts during incubation. The blackbird is a potential but rarely parasitized host of the common cuckoo (*Cuculus canorus*; cuckoo hereafter; Grim et al. 2011) and has been frequently used as a model species in egg-rejection experiments (e.g. Polačiková and Grim 2010; Grim et al. 2011; Samas et al. 2011, 2014; Martín-Vivaldi et al. 2012; Ruiz-Raya et al. 2015, 2016; Soler et al. 2015, 2017), providing us detailed information about their response to experimental foreign eggs. The highly effective anti-parasitic defences showed by blackbirds likely explain the current absence of brood parasitism in this species (Ruiz-Raya et al. 2016). In this study, we first assessed how hosts adjust their baseline hormonal levels when they have to cope with non-mimetic eggs (after egg recognition). We predicted that parasitized females will show higher baseline CORT levels than non-parasitized individuals given the important role of glucocorticoids in the response of birds to environmental stressors (Sapolsky et al. 2000; Landys et al. 2006). Prolactin levels are predicted to remain unaffected in parasitized blackbirds given that previous studies performed in this species have shown the absence of nest desertion (i.e. maintenance of parental effort), even after recognizing non-mimetic parasitic eggs (Soler et al. 2015). Furthermore, an increase in CORT levels might affect body reserves (Sapolsky et al. 2000), which would result in a worse body condition of parasitized females. Second, we studied whether brood parasitism affects the hosts' hormonal stress response since it has been experimentally proven that birds can modulate their stress response according to the value of their current reproduction (Lendvai et al. 2007). We expected that the magnitude of the CORT and prolactin response to the standardised stress protocol is higher in parasitized females as their potential reproductive value is thought to be lower compared to non-parasitized birds given the high fitness costs associated to brood parasitism (Davies 2000). Finally, we investigated whether CORT and prolactin are correlated in blackbirds to provide new information on the potential relationship between these two hormones under different ecological contexts: if CORT and prolactin are functionally related in blackbirds, they should be correlated and modulated according to the same factors (Angelier et al. 2013).

Materials and methods

Study system and experimental procedure

This study was conducted in the Valley of Lecrín (Southern Spain, 36° 56′ N, 3° 33′ W) from late March to May 2015. Since the beginning of the breeding season, we actively searched for blackbird nests in the study area. Once a nest was located, we checked it to determine its content and visited each nest every 2 days to obtain data on laying date and clutch size. We created two experimental groups of nests: parasitized and non-parasitized nests. In the first one, after onset of incubation, blackbird nests were experimentally parasitized by introducing a non-mimetic model egg. In non-parasitized nests, we followed the same procedure, but without introducing the experimental egg. In our study population, the clutch size of blackbirds varies from 2 to 4 eggs (mean ± SE: 2.8 ± 0.12 eggs; Ibáñez-Álamo and Soler 2010). Most nests contained three eggs (74% of nests), whereas nest containing two or four eggs was less frequent during the field season: 18% and 8% of nests, respectively. There was no difference in body condition between blackbird females whose nest contained two, three, or four eggs ($t = -0.51$; $p = 0.61$). Thus, we decided not to use those nests containing four eggs in order to not exceed their maximum natural clutch size in the population. We conducted a sequential assignment of nests to the parasitized or non-parasitized group to control for possible differences in the hormonal levels of the females prior to the experiment. As model eggs, we used real common quail (*Coturnix coturnix*) eggs (weight mean ± SE: 12.2 ± 0.04 g; size: $32.6 \pm 0.1 \times 25.3 \pm 0.1$ mm; $n = 49$), which are slightly larger than blackbird eggs (weight: 6.6 ± 0.1 g; size: $30.4 \pm 0.2 \times 21.1 \pm 0.1$ mm; $n = 40$). This difference hinders the action component of egg ejection thus extending the period of time the parasitic egg is present in the nest (3 days; see below). The addition of a slightly larger egg to the blackbird nests does not hamper the incubation behaviour of this species in our population, neither in this study nor in previous ones (Soler et al. 2015, 2017). Model eggs were coloured red with acrylic paint the day before being placed in the blackbird nest to ensure egg recognition. Experimental eggs painted red have been widely used in egg-rejection studies and acknowledged as good non-mimetic model eggs (e.g. Soler and Møller 1990; Avilés et al. 2004; Martín-Vivaldi et al. 2012) easily detected as a parasitic egg by blackbirds (Ruiz-Raya et al. 2015; Soler et al. 2015). In our study, no blackbird deserted the nest within 3 days after the experimental parasitism, suggesting that the stimulus provided by the non-mimetic egg was not strong enough to elicit substantial behavioural changes. Each model egg was used only in one trial and discarded afterwards. All nests were inspected daily to look for possible cracks or broken eggs (ejection costs).

Blood sampling

Blood samples were taken both from non-parasitized and experimentally parasitized females. Three days after the start of the experiment, all females were captured just after sunrise by using a mist net placed near the nest. We took the blood samples after a period of 3 days mainly because of two

reasons: first, CORT-induced changes in prolactin levels happen progressively and during several days before returning to the initial levels (Angelier et al. 2009a). Secondly, we used a time frame wide enough to detect possible relationships between CORT and body condition. To assess the hormonal levels of blackbirds as well as their hormonal response to stress, females were bled following the standardized technique described by Wingfield (Wingfield 1994). Immediately after capture, an initial blood sample (400–500 μl) was collected from the brachial vein with a 25-gauge needle and 80-μl heparinized microhematocrit tubes (baseline levels). We quantified the time elapsed between the capture and the end of the initial blood sampling to control for the possible effect of handling time on hormone levels, especially for CORT. Thus, all blood samples used in CORT assays were taken within 3 min after capture. After the first bleeding, females were kept in an individual cloth bag and suspended off the ground. For stress-induced hormonal levels, a second blood sample was taken from the same individual 30 min after capture from the brachial vein of the other wing. This period of time has been previously found to result in the maximal stress-induced CORT levels for several species (Mark and Rubenstein 2013), including blackbirds (Partecke et al. 2006; Adams et al. 2011). During the handling time, eggs were covered with cotton to reduce the heat loss. Individuals were marked with individual rings to be certain of using each female only once. All birds were released near the nest in 5–15 min after the last blood sampling and returned to the nest to resume incubation within the next hour (FRR pers. obs.). Blood samples were kept cold and were centrifuged at 4500 RCF for 3 min as soon as possible (maximum 5 h after collection). Plasma was separated and stored at − 20 °C until the hormonal assay. It was not possible to record data blind because our study involved focal animals in the field.

Body condition

After bleeding, all females were weighed twice to the nearest 0.1 g and tarsus length was measured twice to the nearest 0.01 mm. In all cases, measures were made by the same researcher (FRR). We calculated the average weight and tarsus length for each female to estimate the body condition for each individual. Thus, we calculated the "scaled mass index" (SMI) following (Peig and Green 2009, 2010) for each individual. Unlike residual index, which assumes isometry (i.e. proportion of body components remain constant as the size increases), the SMI considers allometry (i.e. relative length of different body parts changing with size) by estimating a scaling exponent and including it in the calculation, which allows to determine the structural effects of growth on mass-length relationships (Peig and Green 2009, 2010).

Hormone assays

All hormonal analyses were performed at the Centre d'Études Biologiques de Chizé (CEBC-CNRS, Villiers en Bois, France). Plasma concentrations of CORT were determined after diethyl-ether extraction by a radioimmunoassay (RIA) as detailed in Lormée et al. (2003). Plasma concentrations of prolactin were also determined using a heterologous RIA assay following the method described by Cherel et al. (1994) and validated for blackbirds (Préault et al. 2005). All samples were run in one assay for both hormones (intra-assay variations, CORT: 10.8%; prolactin: 4.8%). The minimal detectable CORT and prolactin levels were 0.5 and 6 ng/ml, respectively, and no samples fell below these limits.

Statistical analyses

To examine the effect of our manipulation on CORT levels, we performed a generalized linear mixed model (GLMM) with a gamma distribution and a log-link function using the *glmmADMB* R package (Skaug et al. 2016). For prolactin, we fitted a linear mixed model (LMM) using the *nlme* package in R (Pinheiro et al. 2014). Both CORT and prolactin models included the following fixed factors: experimental parasitism (parasitized/non-parasitized), sample time (baseline/stress-induced), the interaction between experimental parasitism and sample time, clutch size and day of incubation in which the experimental egg was introduced (to control for the incubation state of nests). We also included female identity as random factor as the same individuals were sampled twice during the stress-induced protocol. Stepwise procedures were avoided to minimize the chances for type I errors, so full models (i.e. the model containing all the parameters of interest) were used for inference (Whittingham et al. 2006; Mundry and Nunn 2009). We calculated Tukey's HSD as a post hoc test where appropriate from the package *lsmeans* (Lenth 2016). To quantify the proportion of variance explained for our mixed models, we calculated R^2 values as described by Nakagawa and Schielzeth (2013) and Nakagawa et al. (2017). Briefly, we obtained two values of R^2: the marginal R^2 (R^2m), which describes the proportion of variance explained by the fixed factors alone, and the conditional R^2 (R^2c), which describes the proportion of variance explained by both the fixed and random factors.

The effect of our manipulation on the body condition of females was analysed by a linear model including experimental parasitism, clutch size and day of incubation as predictors. Finally, we also examined whether CORT levels, prolactin levels and body condition were correlated regarding both baseline and stress-induced hormonal levels. Model validation of all models was performed by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. All analyses and

graphs were performed using R version 3.2.3 (R Core Team 2014).

Data availability

The datasets used in the current study are available upon request to the corresponding author.

Results

We carried out our experiment in 46 blackbird nests. In seven cases, the experimental egg was ejected from the nest during the day before the blood sampling, so these females were not sampled as their hormone levels could have returned to values similar to those prior to our manipulation (see Ethical note). Furthermore, some females were excluded from the hormonal assays given that the bleeding time exceeded 3 min ($N = 5$). Therefore, we finally report results from 34 blackbird females: 18 parasitized and 16 non-parasitized. We found no ejection costs in this study.

Corticosterone

Overall, parasitized females showed significantly higher CORT levels compared to non-parasitized ones ($\chi^2 = 6.84$; $df = 1$; $p = 0.009$), but these differences depended on the sample time ($\chi^2 = 5.46$; $df = 1$; $p = 0.019$). Specifically, baseline CORT levels of parasitized females were higher than those of non-parasitized birds ($z = -2.63$; $p = 0.04$; Fig. 1a). However, we found no differences between parasitized and non-parasitized females for stress-induced CORT levels ($z = 0.09$; $p = 0.99$; Fig. 1a). CORT levels significantly increased in response to the standardized stress protocol ($\chi^2 = 310.28$; $df = 1$; $p < 0.001$). Clutch size, body condition or incubation day did not affect CORT levels (all cases $p > 0.61$; Table 1). Our statistical model explained 90% of variance, in which the fixed part explained 87% of variance (Table 1).

Prolactin

Experimental parasitism, clutch size, body condition, incubation day or treatment did not affect prolactin levels (all cases $p > 0.48$; Table 1). Prolactin levels significantly decreased in response to the standardized stress protocol ($F_{1, 32} = 141.27$; $p < 0.0001$). However, the decrease in prolactin levels was more pronounced in parasitized females ($F_{1, 32} = 4.43$; $p = 0.04$; Fig. 1b). Thus, we did not find differences between parasitized and non-parasitized females neither regarding baseline ($t = -0.40$; $p = 0.40$) nor stress-induced prolactin levels ($t = 0.80$; $p = 0.86$; Fig. 1b). Our statistical model explained 86% of variance, in which the fixed part explained 35% of variance (Table 1).
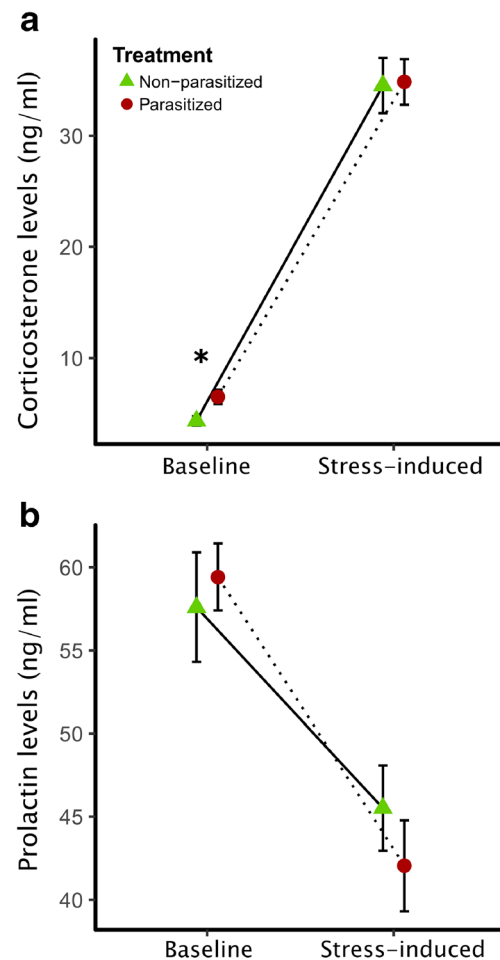

Fig. 1 Influence of the experimental treatment on the hormonal levels of hosts. Baseline and stress-induced CORT (**a**) and prolactin (**b**) levels for parasitized and non-parasitized blackbird females. Asterik indicates significant differences at $p < 0.05$. Data are expressed as means ± SE

We found no correlation between prolactin and CORT levels either for baseline (parasitized females: Pearson's $r = -0.11$, $p = 0.66$; non-parasitized females: Pearson's $r = -0.13$, $p = 0.64$) or stress-induced levels (parasitized females: Pearson's $r = 0.03$, $p = 0.92$; non-parasitized females: Pearson's $r = 0.14$, $p = 0.63$).

Body condition

Experimental parasitism significantly affected body condition of blackbird females ($F_{1, 27} = 10.13$; $p = 0.004$) and parasitized individuals showed a worse body condition (SMI = 93.69 ± 1.06) than non-parasitized ones (SMI = 98.81 ± 1.01; estimate ± SE = −6.32 ± 2.82; $t = -2.43$; $p = 0.03$). Clutch size or incubation did not affect body condition (all cases $p > 0.3$). Our statistical model explained 23% of variance.

We found a negative correlation between baseline CORT levels of parasitized females and body condition: parasitized females with higher CORT levels showed poorer body condition (Pearson's $r = -0.50$, $p = 0.04$; Fig. 2). However, we did

Table 1 Summary of linear mixed models for the effects of treatment and covariates on hormonal levels of blackbird females

	CORT				Prolactin			
	Estimate ± SE	df	χ	p	Estimate ± SE	df	F	P
Intercept	1.902 ± 1.076				46.869 ± 44.960			
Parasitism	0.358 ± 0.136	1	6.84	0.009	1.765 ± 4.417	1,29	0.05	0.812
Sample time	2.072 ± 0.117	1	310.28	< 0.001	− 12.094 ± 1.826	1,32	141.27	< 0.001
Parasitism * sample time	− 0.377 ± 0.162	1	5.46	0.019	− 5.281 ± 2.510	1,32	4.43	0.043
Body condition	− 0.005 ± 0.010	1	0.25	0.614	0.049 ± 0.437	1,31	0.01	0.964
Clutch size	0.045 ± 0.117	1	0.15	0.698	3.478 ± 4.728	1,30	0.52	0.478
Incubation day	0.008 ± 0.026	1	0.08	0.776	0.485 ± 1.048	1,30	0.21	0.647
R^2m	0.881				0.354			
R^2c	0.896				0.860			

not find any relationship between body condition and CORT levels for non-parasitized females (Pearson's $r = 0.25$, $p = 0.36$, Fig. 2).

Regarding prolactin, body condition did not show any relationship with baseline prolactin levels for non-parasitized (Pearson's $r = -0.39$, $p = 0.13$) or parasitized females (Pearson's $r = 0.05$, $p = 0.86$). However, those individuals with a poorer body condition suffered a higher decrease in their prolactin levels in general (Pearson's $r = 0.66$; $p < 0.0001$) as well as within experimental treatment (non-parasitized females: Pearson's $r = 0.53$; $p = 0.03$; parasitized females: Pearson's $r = 0.65$; $p = 0.001$; Fig. 3).

Discussion

Relationships between brood parasitism and hormones at the incubation stage have received little attention so, through this study, we investigated the potential effects of the presence of a non-mimetic egg on the physical and hormonal status of the host. Our study presents five main findings: first, we found that parasitized females showed higher baseline CORT levels than non-parasitized females. Second, experimental brood parasitism significantly reduced the physical state of females. Third, baseline prolactin levels were not affected by the experimental parasitism. Fourth, the prolactin response to stress was more pronounced in experimentally parasitized females. Finally, CORT and prolactin appear to be functionally unrelated in the Eurasian blackbird.

Corticosterone and body condition

Our study shows that the presence of a non-mimetic egg significantly affected the hormonal state of parasitized females in line with previous studies that also found an effect of brood parasitism on CORT levels during the chick and fledgling stage (Ibáñez-Álamo et al. 2012; Mark and Rubenstein
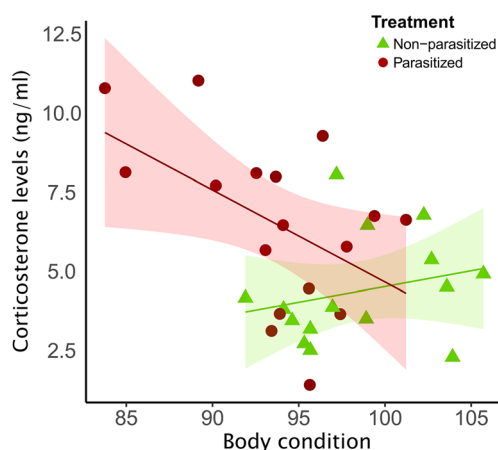

Fig. 2 Baseline CORT levels and body condition. Relationship between baseline CORT levels and body condition (SMI) for parasitized and non-parasitized blackbird females. Shade areas represent 95% confidence intervals
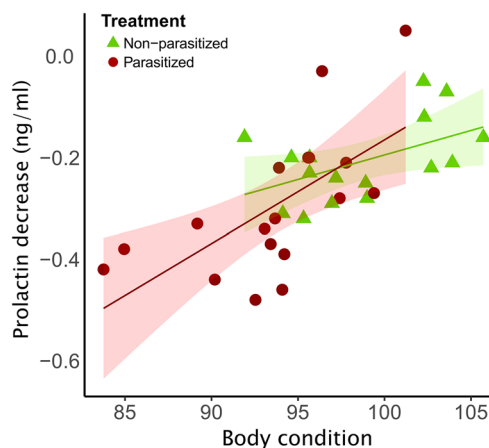

Fig. 3 Prolactin response to stress and body condition. Relationship between the decrease in prolactin levels in response to the stress-induced protocol and body condition (SMI) for parasitized and non-parasitized blackbird females. Shade areas represent 95% confidence intervals

2013). We found higher baseline CORT levels in parasitized female blackbirds compared to non-parasitized individuals (Fig. 1a). These differences might be associated to egg recognition: given that blackbirds easily recognize non-mimetic eggs (e.g. Samas et al. 2011; Martín-Vivaldi et al. 2012; Ruiz-Raya et al. 2015; Soler et al. 2017), egg recognition might trigger changes in the hormonal profile of parasitized females to deal with the parasitic egg. As found in previous studies, egg recognition induces several behavioural changes in blackbirds related to an increase in female activity at the nest, such as the number of touches given to the eggs or the time spent on nest checking (Ruiz-Raya et al. 2016; Soler et al. 2017). These behaviours likely involve an additional energy demand for parasitized females which could be satisfied in the short term by using body reserves. CORT is a well-known mediator in metabolic processes affecting the energetic state of individuals by mobilizing energy from body reserves to deal with environmental stressors (Sapolsky et al. 2000; Landys et al. 2006). In our study, parasitized females showed a worse body condition than non-parasitized ones, probably due to protein or lipid mobilization from storage sites linked to baseline CORT levels rising. In fact, we found a significant negative correlation between CORT levels of parasitized females and their body condition in which higher values of CORT levels corresponded to parasitized females with a worse body condition (Fig. 2). Altogether, our results indicate that the presence of a non-mimetic egg in the blackbird nest resulted in a worse body condition of parasitized females, revealing potential costs of brood parasitism that, to the best of our knowledge, were unknown.

Alternatively, more elevated CORT levels showed by experimentally parasitized females might also be related by other factors unrelated to brood parasitism, such as increased energetic costs linked to the incubation of enlarged clutches. The relationship between clutch size and the energetic state of incubating birds is still poorly known although enlarged clutches have been previously associated to an increase in the "incubation metabolic rate" (reviewed in Nord and Williams 2015), which therefore could lead to an elevation in CORT levels. However, this increase in metabolic rate usually occurs when birds incubate below their lower critical temperature (Nord and Williams 2015). Most importantly, studies on the energy expenditure during incubation show scarce evidence of the effect of experimental manipulations of clutch size on "field metabolic rate" during incubation (Nord and Williams 2015). Furthermore, clutch size did not affect CORT levels in our blackbird population suggesting that this factor alone is unlikely to explain our results. Interestingly, results found by Mark and Rubenstein (2013) showing the absence of physiological costs during the incubation stage in naturally parasitized females of the rufous-and-white wren (*Thryophilus rufalbus*) also suggest that the extra parental care needed for the incubation of an additional

parasitic egg does not seem to be high enough to modify the baseline CORT levels of parasitized individuals. Instead, these results could be explained by the low recognition rates of parasitic eggs exhibited by their model species (Mark 2013).

Standardized stress protocol resulted in a quick and robust increase of CORT levels both in parasitized and non-parasitized females, which has been also found in common host species of brood parasites (Mark and Rubenstein 2013). Strong CORT response to stress has been previously described in several bird species (e.g. Angelier et al. 2013, 2015; Krause et al. 2014), including blackbirds (Partecke et al. 2006; Adams et al. 2011; Ibáñez-Álamo et al. 2011). Despite this, some species can down-regulate their response to acute stressors as a hormonal tactic to optimize their current reproductive effort, even if this involves survival costs (Jessop 2001; O'Reilly and Wingfield 2001; Wingfield and Sapolsky 2003). In our study, non-parasitized females were expected to show a lower adrenocortical stress response as the value of their offspring would be higher compared to parasitized females; however, both groups reached CORT levels nearly six-fold higher than baseline levels. Furthermore, we found no differences in the stress-induced CORT levels of parasitized and non-parasitized females, contrary to what might be expected according to the potential value of their current reproduction (Lendvai et al. 2007). The maintenance of a robust stress response in both groups is expectable from an "emergency life-history stage", a strategy that would allow individuals to minimize the risk of mortality and ensure the future reproduction when dealing with unpredictable perturbations, such as an encounter with a predator or our capture stress protocol (Wingfield et al. 1998).

Prolactin

Baseline prolactin levels of parasitized females remained unaffected despite the presence of a non-mimetic parasitic egg in the nest (Fig. 1b). Prolactin has been classically proposed as the hormone responsible for the expression of parental care in birds (Silver 1984; Hall et al. 1986; Sharp et al. 1988; Buntin 1996; Lormée et al. 2000; Sockman et al. 2006) and has been suggested to be a good proxy of parental effort (i.e. amount and quality of parental behaviour) (Angelier and Chastel 2009). In fact, elevated prolactin levels are positively related to breeding success through a greater parental effort (Miller et al. 2009), while decreasing prolactin levels are often linked to processes such as incubation interruption and nest desertion (Cherel et al. 1994; Chastel and Lormée 2002; Angelier et al. 2007, 2015; Groscolas et al. 2008; Spée et al. 2010). Unaffected prolactin levels exhibited by parasitized females in our study could thus explain previous findings showing absence of nest desertion as an egg-rejection strategy in blackbirds (Soler et al. 2015). Therefore, more studies are needed to investigate whether this is a general physiological mechanism

responsible for the absence of nest desertion in host species, and the potential role that this hormone might play in other life stages (i.e. chick or fledgling stages).

Unlike CORT, we found no correlation between prolactin levels and body condition in both parasitized and non-parasitized blackbirds. In some species such as capital breeders, which rely on stored energy for reproduction, it is expected that prolactin is negatively affected by body condition as individuals with insufficient body reserves would benefit by redirecting energy away from reproduction (O'Dwyer et al. 2006). However, blackbirds exercise a behavioural control on their stored energy and they do not rely on large body reserves during reproduction, when foraging opportunities are greater (Macleod et al. 2005), which would explain why baseline prolactin levels are not affected by the low body condition of parasitized females.

Prolactin levels of both parasitized and non-parasitized blackbirds were affected by the standardised stress protocol, which led to a significant decrease of baseline levels in both groups (prolactin response to stress; Fig. 1b). Prolactin response to stress is considered an adaptive mechanism in birds to cope with stressors allowing them to disrupt the current parental care under environmental constraints to maximize future reproduction (Chastel et al. 2005). Indeed, the magnitude of the prolactin response to stress has been suggested to reflect the individual's degree of parental investment (Angelier and Chastel 2009). In agreement with our prediction, parasitized females showed a more pronounced prolactin response to stress than non-parasitized females, which might indicate that the presence of non-mimetic eggs impacts on the parental investment of blackbird females. This investment, however, does not seem to be affected in our population given the absence of nest desertion, but a detailed study on other parental care behaviours would help to detect more subtle behavioural effects of this endocrine change. According to the "brood value hypothesis", individuals are able to modulate their stress response with respect to the value of current reproduction (Lendvai et al. 2007; Lendvai and Chastel 2008). Brood parasitism usually involves important fitness costs on hosts (Davies 2000), so parasitized females may be expected to markedly reduce their parental care to redirect some of the energy available from reproduction to survival in response to stressful situations. This response may be especially important in cases where the energy availability is limited, and previous studies have shown that circulating prolactin levels are negatively impacted from a certain threshold of body condition as individuals energetically constrained usually show a sharper and quicker decline of prolactin levels (Cherel et al. 1994; Criscuolo et al. 2002; O'Dwyer et al. 2006; Groscolas et al. 2008; Angelier et al. 2009b; Spée et al. 2010; Riechert et al. 2014). Most of these studies have focused on species with large body reserves such as seabirds, but little is known about smaller species (i.e., passerines) that do not rely on large body

reserves for reproduction (reviewed in Angelier et al. 2016a). Our study provides one of the few pieces of evidence supporting this relationship for passerines as we found that blackbirds with a poorer body condition experienced a more pronounced decline of prolactin levels in response to stress (Fig. 3).

Relationships between CORT and prolactin

In this study, we found no correlation between CORT and prolactin levels indicating that these hormones are not linked in the Eurasian blackbird. This result fits with previous studies showing that these hormones are usually unrelated in birds (Angelier et al. 2013, 2016b; Krause et al. 2015). The CORT response to stress is known to be associated with physiological changes aimed to redirect energy to cope with stressors (Sapolsky et al. 2000; Landys et al. 2006), which can be important when dealing with parasitic eggs. On the other hand, the prolactin response to stress is linked to parental decision when dealing with environmental perturbations (Angelier and Chastel 2009), so it could govern some host responses such as the desertion of parasitized clutches. The combined study of both hormones in future studies will provide valuable information to understand some crucial aspects of the anti-parasitic host responses, such as the energetic state of hosts and its potential impact on parental decisions.

In conclusion, our results show that the presence of a non-mimetic parasitic egg modifies the baseline CORT levels of blackbird females, alter their hormonal response to stress and can negatively affect their body condition. This study provides therefore the first evidence showing that non-mimetic parasitic eggs can influence the endocrine profiles of hosts and contributes to expand our knowledge on the still poorly understood physiological effects of brood parasitism. The study of hormones related to the maintenance of parental care in birds can be crucial to understand some anti-parasitic responses previously found in several host species, such as acceptance decisions or the absence of nest desertion. Taken together, these results highlight the need of additional studies focused on the endocrine mechanisms behind the life-history decisions of bird species currently impacted by brood parasitism.

Compliance with ethical standards

Ethical approval   We performed the study following all relevant Spanish national (Decreto 105/2011, 19 de Abril) and regional guidelines. Ethical approval for this study was not required. Research disturbance, due to blood sampling protocol (details provided above), was minimized by using only those females that did not ejected the parasitic egg. The time spent at each nest was the minimum necessary for blood sampling. No female deserted their nest during the 3 days after to our experimental manipulation and none exhibited any long-term effects of the study.

Competing interests   The authors declare that they have no conflict of interest.

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