Physiological stress responses to non-mimetic model brood parasite eggs: leukocyte profiles and heat-shock protein Hsp70 levels

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Abstract

Obligate avian brood parasites lay their eggs in the nest of other bird species (hosts). Brood parasitism often imposes severe fitness costs on hosts, which selects for the evolution of effective anti-parasitic defences, such as recognition and rejection of brood parasite eggs. Glucocorticoids have been recently found to mediate host physiological and behavioural adjustments in response to brood parasite eggs; however, it remains unclear whether brood parasitism triggers a general response involving multiple physiological elements. In this study, we experimentally investigated whether a salient brood parasitic stimulus (the presence of a non-mimetic model egg in the nest) causes physiological adjustments in adult Eurasian blackbirds (*Turdus merula*) at immune (leukocyte profiles) and cellular (heat-shock protein Hsp70 synthesis) level. Also, we explored whether these physiological changes are mediated by variations in corticosterone levels. We found that experimental brood parasitism caused an increase in heterophils and a decrease in lymphocytes, leading to higher H/L ratios in parasitized birds. Nevertheless, we did not find trade-offs between immune function and corticosterone levels. Hsp70 synthesis was not affected by our experimental manipulation. Our findings provide evidence that brood parasite eggs trigger a general stress response in egg-rejecter hosts, including changes in cellular immune profiles.

Keywords: Avian brood parasitism, corticosterone, Eurasian blackbird, H/L ratio.

INTRODUCTION

Obligate avian brood parasites, which account for approximately 1% of bird species (Mann, 2017), lay their eggs in the nests of heterospecific birds (hosts), taking advantage of the parental care that hosts provide to their young (Payne, 1977). Interspecific brood parasitism imposes significant fitness costs on hosts, which selects for the evolution of anti-parasitic host defences (e.g., the recognition and rejection of parasitic eggs; Feeney et al., 2014; Soler, 2014) and ultimately can lead to co-evolutionary arms races between brood parasites and hosts (Rothstein, 1990). Over the past decades, much research has focused on the ecological and behavioural aspects of avian brood parasite-host interactions (Soler, 2017). However, the physiological mechanisms underlying host responses to brood parasitism have received comparatively little attention despite the fact that brood parasitism may potentially trigger significate adjustments in host physiology, which can have important consequences for the expression and evolution of key anti-parasitic defences such as egg rejection (Abolins-Abols and Hauber, 2018; Avilés, 2018; Ruiz-Raya, 2021).

Previous studies on host physiological responses to brood parasitism have focused primarily on the endocrinology of stress. Glucocorticoid hormones are known to mediate allostasis in vertebrates, triggering physiological and behavioural changes that help individuals to cope with environmental challenges (Breuner et al., 2008; Wingfield et al., 1998), including avian brood parasitism (Abolins-Abols and Hauber, 2020). Brood parasitism stimuli is known to increase corticosterone (CORT) levels in adult hosts during incubation (Ruiz-Raya *et al.* 2018; but see Scharf *et al.* 2021) and nestling stages (Antonson et al., 2020). Parasitized birds also show elevated CORT responsiveness to stressors during the fledgling period, which can lead to detrimental long-term consequences (Mark and Rubenstein, 2013). Theory predicts that physiological responses to stress will operate at different levels and include multiple physiological systems working together (Wingfield and Romero, 2015). Thus, characterizing the stress response to avian brood parasitism will require assessing different physiological biomarkers that provide supplementary information on the nature of these physiological adjustments (Breuner et al., 2013; MacDougall-Shackleton et al., 2019; O'dell et al., 2014).

A crucial aspect of host physiology that could be affected by brood parasitism is immune function. Environmental stressors are known to cause changes in the relative proportion

of white blood cell types (i.e. leukocytes; Davis et al., 2008), a highly conserved physiological response in vertebrates. This parameter has become a widely applied tool in ecophysiology to assess individual responses to stress (Davis et al., 2008; O'dell et al., 2014). The relative proportion of heterophils and lymphocytes (H/L ratio), the two most abundant white cell types in birds, is known to increase in response to external stressors such as climatic conditions, parasites or social challenges (Davis et al., 2008; Minias, 2019; Minias et al., 2018). These stress-induced changes in leukocyte number are typically slower and last longer (from one hour to days) than rapid CORT responses, making leukocyte biomarkers particularly informative for obtaining measures of chronic environmental stress (Davis and Maney, 2018; O'dell et al., 2014). Importantly, short-term changes in H/L ratios may be mediated by glucocorticoids (Sapolsky et al., 2000), although stress hormones and leukocyte profiles are not always correlated (Davis and Maney, 2018). Previous studies have shown that rearing brood parasitic nestlings may cause reduced humoral immune responses in hosts (Antonson et al., 2020), yet the effects of brood parasitism on the components of cell-mediated immunity are still unknown.

Other biomarkers, such as heat-shock proteins (Hsp), in particular the Hsp60 and Hsp70 families, have been widely used to assess long-term chronic stress in wild bird populations (Herring and Gawlik, 2007; O'dell et al., 2014). Hsp are molecular chaperones involved in cellular 'house-keeping' functions, whose expression is induced to protect cells from damage caused by a wide range of stressors associated with parasites, environmental or social challenges (Martínez-Padilla et al., 2004; O'dell et al., 2014; Sørensen et al., 2003). This provides valuable supplementary information to hormonal and immune indicators (O'dell et al., 2014). Hsp expression is thought to be part of a general stress response (Asea and Kaur, 2018), and may be associated with glucocorticoid levels (Asea and Kaur, 2018; Mahmoud et al., 2004). The combined use of different biomarkers may therefore help to elucidate the nature and timing of host stress responses; however, there is still little information on the effect of avian brood parasitism on leukocyte profiles and stress protein expression in adult hosts.

Here, we investigate whether a salient brood parasitism stimulus (the presence of one parasitic egg in the nest) triggers significant adjustments in host physiology. Specifically, we evaluated different biomarkers of physiological stress at the immune (leukocyte profile) and cellular level (Hsp expression) in experimentally parasitized and non-parasitized adult hosts. We predict that if the presence of a non-mimetic brood parasite

egg induces a general stress response in adult hosts, then we will find elevated H/L ratios and increased Hsp70 expression caused by experimental parasitism. Additionally, we take advantage of our own data on the glucocorticoid response to experimental brood parasitism (from the same individuals, Ruiz-Raya et al., 2018) to explore, through structural equation modelling, whether the effects of experimental brood parasitism on H/L ratios and Hsp70 expression are mediated indirectly by variations in plasma CORT.

MATERIAL AND METHODS

Study system

Our study was conducted in a Eurasian blackbird (*Turdus merula*) population located in the Valley of Lecrín, Spain, from March to May 2015. The Eurasian blackbird (*Turdus merula*, hereafter blackbird) is an occasional common cuckoo (*Cuculus canorus*) host frequently used in brood parasitism studies (see e.g., Grim et al., 2011; Roncalli et al., 2019; Ruiz-Raya et al., 2015; Samas et al., 2011; Soler et al., 2015; Soler et al., 2017). Female blackbirds, the sex responsible for egg rejection in this species (Ruiz-Raya et al., 2019), show fine-tuned egg-recognition abilities (see references above).

Field procedure

From the beginning of the breeding season, we located active blackbird nests, which were visited every two days to obtain data on laying date and clutch size. The day after clutch completion, breeding pairs were randomly selected to incubate clutches either with (parasitized group, n =18) or without non-mimetic parasitic model eggs (non-parasitized control group, n = 16). Following a previously established methodology, parasitic models eggs were painted red to simulate non-mimetic eggs (Avilés et al., 2004; Martín-Vivaldi et al., 2012; Roncalli et al., 2017; Soler and Møller, 1990), which are easily detected by blackbirds (Ruiz-Raya et al., 2019, 2015; Soler et al., 2015). As model eggs, we used natural (commercial) common quail (*Coturnix coturnix*) eggs (32.6 ± 0.1 x 25.3 ± 0.1 mm; n = 49) slightly larger than blackbird eggs (30.4 ± 0.2 x 21.1 ± 0.1 mm; n = 40), a type of model egg previously used to elicit egg recognition in blackbirds (Ruiz-Raya et al., 2018; Soler et al., 2017). In our study population, blackbird clutch size varies from 2 to 5 eggs (Ibáñez-Álamo and Soler 2010), but we only used nests containing 2 or 3 eggs to avoid exceeding the maximum natural clutch size after experimental parasitism. No

blackbird ejected the parasitic model egg or deserted the nests by the end of brood parasitism trials.

72 hours after the introduction of the parasitic model egg, all focal females were captured (6:00 – 8:00 am) by using a mist net placed near the focal nest (1 - 5 m). Such 72-hours period has been proved to be a time frame suitable to assess sustained physiological changes in response to experimental brood parasitism (Ruiz-Raya et al., 2018). Immediately after capture (< 3 min), a blood sample (400-500 µl) was collected from the brachial vein with a 25-gauge needle and 80 µl heparinized microhematocrit tubes. Additionally, a drop of blood was transferred to a slide to make one-cell-layer blood smears from both parasitized and control females. Smears were air-dried and stored in darkness until methanol fixation. All females were marked with individual rings and released near the nest 5-15 minutes after blood sampling. In all cases, experimental females returned to the focal nest to resume incubation within the next hour (as revealed by warm clutches). Blood samples were kept cold and, once in the lab, centrifuged at 4500 RCF for 3 min (max. 4 hours after collection). Plasma and red blood cells (RBC) were separated and stored at −20 °C until laboratory assays. Blood smears were fixed in methanol (Houwen, 2002; O'dell et al., 2014).

Laboratory analyses

Blood smears were stained by using the Giemsa method and scanned, blind to the treatment, at 1000× magnification under a light microscope. Following a general protocol for leukocyte characterization is slides (O'dell et al., 2014), we counted a random sample of 100 leukocytes from each blood smear, and classified them into heterophils (H), lymphocytes (L), and other leukocyte types (i.e., basophils, eosinophils and monocytes) according to the criteria of Hawkey et al., (1989). Then, the H/L ratio was then calculated for each individual by dividing the number of heterophils by the number of lymphocytes. All blood smears were assessed by the same researcher (RV) to reduce variability. Additionally, twenty-five randomly chosen smears were assessed twice to estimate repeatability of H/L ratio measurements, confirming that leukocyte count was highly repeatable (intra-class correlation coefficient, ICC = 0.86, $p < 0.001$). Hsp70 expression was quantified from red blood cells at the Ecophysiology Laboratory of the Estación Biológica de Doñana (Spanish National Research Council, Spain) using a commercial ELISA kit (ADI-EKS-700B, ENZO Biochem Inc., Farmengdale, New York) by

following the manufacturer instructions. Total proteins were measured using the Bradford method (Kruger, 1994) and Hsp70 values were corrected according to total protein concentration in the samples. CORT levels were measured from plasma samples by heterologous radioimmunoassay (RIA) following a protocol previously validated for blackbirds (see Ruiz-Raya *et al.* 2018 for additional details on CORT assays).

Statistical analyses

All analyses and graphs were performed using R version 3.6.1 (R Core Team, 2019). We used linear models (LMs) to assess between-groups differences in heterophil (Box-Cox transformed), lymphocyte, H/L ratio (Box-Cox transformed) and Hsp70 levels. All models included the brood parasitism treatment, the clutch size (two/three) and the two-way interaction between these terms.

Structural equation modeling (SEM) was used to examine direct and indirect causal relationships between our brood parasitism treatment, the main biomarker of the leukocyte response to stress (the H/L ratio; O'dell et al., 2014), and Hsp70 expression by using the *piecewiseSEM* package (Lefcheck, 2016). First, we explored direct links between experimental brood parasitism and heterophils, lymphocytes and Hsp70 expression, as well as indirect paths through the links with plasma corticosterone concentration (full model). The final model was selected by using Shipley's extension for the Akaike Information Criteria (AIC; Shipley, 2013) and evaluated its goodness of fit using the Fisher's *C* statistic (Lefcheck, 2016). All models described above satisfied the linearity and homoscedasticity criteria.

RESULTS

Experimentally parasitized females showed a higher number of heterophiles ($F_{1,30} = 8.25$, $p = 0.007$, Fig. 1a), and a lower number of lymphocytes ($F_{1,30} = 10.60$, $p = 0.004$. Fig. 1b), compared to non-parasitized control females. As expected, parasitized females showed a higher H/L ratio than non-parasitized control females ($F_{1,30} = 9.11$, $p = 0.005$, Figure 1c). Neither the clutch size nor its interaction with the experimental treatment had an effect on the components of the cellular immunity (i.e., heterophil and lymphocyte counts) or the H/L ratio ($p > 0.27$ in all cases). Contrary to our prediction, Hsp70

212 expression was not affected by our brood parasitism manipulation ($F_{1,30} = 0.01$, $p = 0.84$,
213 Fig. 1d), independently of clutch size ($F_{1,30} = 0.25$, $p = 0.62$).

214 SEM analyses confirmed that experimental brood parasitism had a large direct positive
215 effect on the H/L ratio (Fig. 2, Table S1), but no indirect effects via CORT were detected
216 (Fig.2, Table S1). As expected, we found a direct positive effect of the brood parasite
217 stimulus (i.e., the presence of a non-mimetic egg in the nest) on plasma CORT
218 concentration (Fig. 2, Table S1).

219

220 **DISCUSSION**

221 We provide evidence that brood parasitic egg stimulus caused significant changes in host
222 leukocyte profiles and, as a result, experimentally parasitized birds showed a higher H/L
223 ratio compared to non-parasitized control individuals. Importantly, these effects were not
224 mediated by plasma glucocorticoid concentration. At the cellular level, our experimental
225 manipulation did not affect the expression of stress proteins. To our knowledge, this is
226 the first evidence of the effects of brood parasitism on the immune status of adult hosts
227 during the incubation phase.

228 We found that the presence of a non-mimetic model egg in the nest caused a significant
229 increase in heterophils and a decrease in lymphocytes, resulting in higher H/L ratios in
230 parasitized birds (Fig. 1). These physiological adjustments could be caused by different
231 factors related to the presence of parasitic eggs in the nest. First, given the high
232 recognition abilities shown by female blackbirds (see e.g., Samas et al., 2011; Ruiz-Raya
233 et al., 2019), changes in immune function could be part of a general stress response
234 triggered by the recognition of foreign eggs. Changes in host physiology in response to
235 brood parasitism may also include variations in glucocorticoid levels (Ruiz-Raya et al.,
236 2018), which can promote anti-parasitic responses (Abolins-Abols and Hauber, 2020).
237 Indeed, it has recently been shown that experimental brood parasitism either with mimetic
238 or non-mimetic eggs does not lead to changes in the physiology of the prothonotary
239 warbler (*Protonotaria citrea*), an egg-accepter host of the brown-headed cowbird
240 (*Molothrus ater*) (Scharf et al., 2021). This reinforces the idea that these physiological
241 adjustments are, at least partially, triggered by egg recognition.

242 However, it is also possible that changes in the H/L ratio are related to increased
243 incubation demands associated with increased clutch size (Davis and Maney, 2018;
244 Hanssen et al., 2005). In our study, the effects of experimental brood parasitism on
245 immune biomarkers were not dependent on clutch size, and previous studies have
246 reported that other indicators of physiological stress, such as CORT levels, remain
247 unaffected in hosts naturally parasitized with mimetic eggs (Mark and Rubenstein, 2013).
248 The results described above suggest that incubation demands associated with an
249 additional (parasitic) egg would cause negligible physiological changes in adult hosts *per*
250 *se*. On the other hand, physiological adjustments triggered by egg recognition and brood
251 enlargement would be expected to act simultaneously during natural brood parasitism
252 events, although some brood parasites may occasionally remove host eggs when visiting
253 target nests (Reboreda et al., 2017). Our study design was unable to assess the separate
254 effects of these factors, so future experimental designs will need to consider alternative
255 manipulations to elucidate the relative importance of egg recognition and brood
256 enlargement in triggering physiological stress responses to brood parasitism, especially
257 in egg-rejecter species with finely tuned egg-recognition abilities.

258 Regarding the link between immune function and glucocorticoids, our findings confirmed
259 previously published data on the direct positive effects of non-mimetic eggs on plasma
260 CORT of adult hosts (Ruiz-Raya et al., 2018). However, variation in plasma CORT did
261 not mediate an indirect effect of brood parasitism on leukocyte profiles (Fig. 2). This is
262 consistent with previous studies showing that glucocorticoid levels (CORT or cortisol)
263 and leukocyte profiles (H/L ratio) are not always correlated in wild vertebrates (reviewed
264 in Davis and Maney, 2018). Individual trade-offs between CORT and immune responses
265 (humoral immunity) also appear to be absent in cowbird hosts rearing parasite chicks
266 (Antonson et al., 2020). The lack of correlation between these two measures of
267 physiological stress may be due to differences in the timing of CORT and leukocyte
268 responses to chronic stressors (Davis and Maney, 2018). Thus, it may be plausible that,
269 while leucocyte responses to brood parasite model eggs may persist for relatively long
270 periods, CORT response decline over time in some individuals.

271 Finally, experimental brood parasitism did not elicit differential physiological responses
272 in terms of Hsp70 levels within three days, and Hsp concentration was not related to
273 variation in CORT levels. The short-term stress associated with brood parasite model
274 eggs during this period of time does not appear to cause rapid up-regulation of stress

proteins, whose synthesis is a reliable indicator of chronic stress (O'dell et al., 2014). Nevertheless, we cannot rule out that the expression of Hsp proteins may be affected in scenarios where brood parasitism is expected to involve sustained stress for adult hosts, for example, during rearing of brood parasite nestlings or fledglings.

In conclusion, our results show that the presence of a non-mimetic brood parasite egg in the nest causes significant changes in the cellular immune profiles of adult hosts. These results, together with previous studies on the glucocorticoid response to brood parasite eggs (Ruiz-Raya et al., 2018), as well as evidence from the nestling and fledgling periods (Antonson et al., 2020; Mark and Rubenstein, 2013), indicate that parasitism triggers a generalized stress response affecting multiple physiological components in adult hosts. We encourage the use of different physiological biomarkers in order to gain a comprehensive view of the host physiological response to avian brood parasitism.

Ethical approval

We performed the study following all relevant Spanish national (Decreto 105/2011, 19 de Abril) and regional guidelines. No female deserted their nest during the 3 days after to our experimental manipulation and none exhibited any long-term effects of the study.

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Conflict of interest

The authors declare no competing interests.

Author contribution

FRR and MS conceived and designed the study. FRR and TA conducted the field work. FRR and RV performed the laboratory work. FRR conducted the data analysis and wrote the first draft. All authors critically contributed to drafts and gave final approval for publication.

References

Abolins-Abols, M., Hauber, M.E., 2020. Endocrine regulation of egg rejection in an avian brood parasite host. Biol. Lett. 16, 20200225. https://doi.org/10.1098/rsbl.2020.0225

Abolins-Abols, M., Hauber, M.E., 2018. Host defences against avian brood parasitism: an endocrine perspective. Proc. R. Soc. B Biol. Sci. 285, 20180980. https://doi.org/10.1098/rspb.2018.0980

Antonson, N.D., Hauber, M.E., Mommer, B.C., Hoover, J.P., Schelsky, W.M., 2020. Physiological responses of host parents to rearing an avian brood parasite: An experimental study. Horm. Behav. 125, 104812. https://doi.org/10.1016/j.yhbeh.2020.104812

Asea, A.A.A., Kaur, P. (Eds.), 2018. Heat shock proteins and stress, Heat Shock Proteins. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-90725-3

Avilés, J.M., 2018. Can hosts tolerate avian brood parasites? An appraisal of mechanisms. Behav. Ecol. 29, 509–519. https://doi.org/10.1093/beheco/arx150

Avilés, J.M., Soler, J.J., Soler, M., Møller, A.P., 2004. Rejection of parasitic eggs in relation to egg appearance in magpies. Anim. Behav. 67, 951–958. https://doi.org/10.1016/j.anbehav.2003.08.022

Breuner, C.W., Delehanty, B., Boonstra, R., 2013. Evaluating stress in natural populations of vertebrates: total CORT is not good enough. Funct. Ecol. 27, 24–36. https://doi.org/10.1111/1365-2435.12016

Breuner, C.W., Patterson, S.H., Hahn, T.P., 2008. In search of relationships between the acute adrenocortical response and fitness. Gen. Comp. Endocrinol. 157, 288–295. https://doi.org/10.1016/j.ygcen.2008.05.017

Davis, A.K., Maney, D.L., 2018. The use of glucocorticoid hormones or leucocyte profiles to measure stress in vertebrates: What's the difference? Methods Ecol. Evol. 9, 1556–1568. https://doi.org/10.1111/2041-210X.13020

Davis, A.K., Maney, D.L., Maerz, J.C., 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. 22, 760–772. https://doi.org/10.1111/J.1365-2435.2008.01467.X

Feeney, W.E., Welbergen, J.A., Langmore, N.E., 2014. Advances in the study of coevolution between avian brood parasites and their hosts. Annu. Rev. Ecol. Evol. Syst. 45, 227–246. https://doi.org/10.1146/annurev-ecolsys-120213-091603

340 Grim, T., Samaš, P., Moskát, C., Kleven, O., Honza, M., Moksnes, A., Røskaft, E.,
341 Stokke, B.G., 2011. Constraints on host choice: why do parasitic birds rarely
342 exploit some common potential hosts? J. Anim. Ecol. 80, 508–518.
343 https://doi.org/10.1111/j.1365-2656.2010.01798.x

344 Hanssen, S.A., Hasselquist, D., Folstad, I., Erikstad, K.E., 2005. Cost of reproduction in
345 a long-lived bird: incubation effort reduces immune function and future
346 reproduction. Proc. R. Soc. B Biol. Sci. 272, 1039–1046.
347 https://doi.org/10.1098/RSPB.2005.3057

348 Hawkey, C.M., Dennett, T.B., 1989. Color atlas of comparative veterinary hematology :
349 normal and abnormal blood cells in mammals, birds and reptiles, 1st ed. ed. Iowa
350 State University Press, Ames [Iowa].

351 Herring, G., Gawlik, D.E., 2007. The role of stress proteins in the study of allostatic
352 overload in birds: use and applicability to current studies in avian ecology.
353 ScientificWorldJournal. 7, 1596–1602. https://doi.org/10.1100/TSW.2007.242

354 Houwen, B., 2002. Blood film preparation and staining procedures. Clin. Lab. Med. 22,
355 1–14. https://doi.org/10.1016/S0272-2712(03)00064-7

356 Ibáñez-Álamo, J.D., Soler, M., 2010. Does urbanization affect selective pressures and
357 life-history strategies in the common blackbird (*Turdus merula* L.)? Biol. J. Linn.
358 Soc. 101, 759–766. https://doi.org/10.1111/j.1095-8312.2010.01543.x

359 Lefcheck, J.S., 2016. piecewiseSEM: Piecewise structural equation modelling in r for
360 ecology, evolution, and systematics. Methods Ecol. Evol. 7, 573–579.
361 https://doi.org/10.1111/2041-210X.12512

362 MacDougall-Shackleton, S.A., Bonier, F., Romero, L.M., Moore, I.T., 2019.
363 Glucocorticoids and "stress" are not synonymous. Integr. Org. Biol. 1.
364 https://doi.org/10.1093/IOB/OBZ017

365 Mahmoud, K.Z., Edens, F.W., Eisen, E.J., Havenstein, G.B., 2004. Ascorbic acid
366 decreases heat shock protein 70 and plasma corticosterone response in broilers
367 (Gallus gallus domesticus) subjected to cyclic heat stress. Comp. Biochem.
368 Physiol. Part B Biochem. Mol. Biol. 137, 35–42.
369 https://doi.org/10.1016/J.CBPC.2003.09.013

370 Mann, C.F., 2017. A taxonomic review of obligate and facultative interspecific avian
371 brood parasitism, in: Avian Brood Parasitism Behaviour, Ecology, Evolution and
372 Coevolution. Springer, Cham, pp. 61–92. https://doi.org/10.1007/978-3-319-
373 73138-4_4

374 Mark, M.M., Rubenstein, D.R., 2013. Physiological costs and carry-over effects of
375 avian interspecific brood parasitism influence reproductive tradeoffs. Horm.
376 Behav. 63, 717–722.

377 Martín-Vivaldi, M., Soler, J.J., Møller, A.P., Pérez-Contreras, T., Soler, M., 2012. The
378 importance of nest-site and habitat in egg recognition ability of potential hosts of
379 the Common Cuckoo Cuculus canorus. Ibis (Lond. 1859). 155, 140–155.
380 https://doi.org/10.1111/ibi.12000

381 Martínez-Padilla, J., Martínez, J., Dávila, J.A., Merino, S., Moreno, J., Millán, J., 2004.

382 Within-brood size differences, sex and parasites determine blood stress protein
383 levels in Eurasian Kestrel nestlings. Funct. Ecol. 18, 426–434.
384 https://doi.org/10.1111/J.0269-8463.2004.00874.X

385 Minias, P., 2019. Evolution of heterophil/lymphocyte ratios in response to ecological
386 and life-history traits: A comparative analysis across the avian tree of life. J. Anim.
387 Ecol. 88, 554–565. https://doi.org/10.1111/1365-2656.12941

388 Minias, P., Włodarczyk, R., Meissner, W., 2018. Leukocyte profiles are associated with
389 longevity and survival, but not migratory effort: A comparative analysis of
390 shorebirds. Funct. Ecol. 32, 369–378. https://doi.org/10.1111/1365-
391 2435.12991/SUPPINFO

392 O'dell, D.A., Carlo, M.A., Kimmitt, A., Bikowski, E., Morris, K.R., Dolby, A., 2014. A
393 comparison of techniques measuring stress in birds. Va. J. Sci. 65.

394 Payne, R.B., 1977. The ecology of brood parasitism in birds. Annu. Rev. Ecol. Syst. 8,
395 1–28.

396 R Core Team, 2019. R: A language and environment for statistical computing. R
397 Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-
398 project.org/.

399 Reboreda, J.C., Fiorini, V.D., De Mársico, M.C., Gloag, R., Scardamaglia, R.C., 2017.
400 Parasitic behaviour of interspecific brood parasitic females, in: Soler, M. (Ed.),
401 Avian Brood Parasitism Behaviour, Ecology, Evolution and Coevolution. Springer,
402 pp. 325–342. https://doi.org/10.1007/978-3-319-73138-4_18

403 Roncalli, G., Ibáñez-Álamo, J.D., Soler, M., 2017. Size and material of model parasitic
404 eggs affect the rejection response of Western Bonelli's Warbler *Phylloscopus*
405 *bonelli*. Ibis (Lond. 1859). 159, 113–123. https://doi.org/10.1111/ibi.12431

406 Roncalli, G., Soler, M., Ruiz-Raya, F., Serrano-Martín, A.J., Ibáñez-Álamo, J.D., 2019.
407 Predation risk affects egg-ejection but not recognition in blackbirds. Behav. Ecol.
408 Sociobiol. 73. https://doi.org/10.1007/s00265-019-2668-x

409 Rothstein, S.I., 1990. A model system for coevolution: avian brood parasitism. Annu.
410 Rev. Ecol. Syst. 21, 481–508.
411 https://doi.org/10.1146/annurev.es.21.110190.002405

412 Ruiz-Raya, F., 2021. Ecophysiology of egg rejection in hosts of avian brood parasites:
413 new insights and perspectives. Curr. Zool. 1–8. https://doi.org/10.1093/cz/zoab042

414 Ruiz-Raya, F., Soler, M., Abaurrea, T., Chastel, O., Roncalli, G., Ibáñez-álamo, J.D.,
415 2018. Hormonal responses to non-mimetic eggs: is brood parasitism a
416 physiological stressor during incubation? Behav. Ecol. Sociobiol. 72.
417 https://doi.org/doi.org/10.1007/s00265-018-2565-8

418 Ruiz-Raya, F., Soler, M., Roncalli, G., Ibáñez-Álamo, J.D., 2019. Egg-recognition
419 abilities in non-incubating males: implications for the evolution of anti-parasitic
420 host defenses. Behav. Ecol. Sociobiol. 73.

421 Ruiz-Raya, F., Soler, M., Sánchez-Pérez, L.L., Ibáñez-Álamo, J.D., 2015. Could a
422 factor that does not affect egg recognition influence the decision of rejection?

PLoS One 10, 1–10. https://doi.org/10.1371/journal.pone.0135624

Samas, P., Hauber, M.E., Cassey, P., Grim, T., 2011. Repeatability of foreign egg rejection: Testing the assumptions of co-evolutionary theory. Ethology 117, 606–619. https://doi.org/10.1111/j.1439-0310.2011.01917.x

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Preparative actions. Endocr. Rev. 21, 55–89. https://doi.org/10.1210/er.21.1.55

Scharf, H.M., Abolins-Abols, M., Stenstrom, K., Tolman, D., Schelsky, W., Hauber, M.E., 2021. Exposure to a mimetic or non-mimetic model avian brood parasite egg does not produce differential glucocorticoid responses in an egg-accepter host species. Gen. Comp. Endocrinol. 304, 113723. https://doi.org/10.1016/j.ygcen.2021.113723

Shipley, B., 2013. The AIC model selection method applied to path analytic models compared using a d-separation test. Ecology 94, 560–564. https://doi.org/10.1890/12-0976.1

Soler, M., 2014. Long-term coevolution between avian brood parasites and their hosts. Biol. Rev. 89, 688–704. https://doi.org/10.1111/brv.12075

Soler, M., Møller, A.P., 1990. Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. Nature 343, 748–750. https://doi.org/10.1038/343748a0

Soler, M., Ruiz-Raya, F., Roncalli, G., Ibáñez-Álamo, J.D., 2017. Relationships between egg-recognition and egg-ejection in a grasp-ejector species. PLoS One 12, e0166283. https://doi.org/10.1371/journal.pone.0166283

Soler, M., Ruiz-Raya, F., Roncalli, G., Ibáñez-Álamo, J.D., 2015. Nest desertion cannot be considered an egg-rejection mechanism in a medium-sized host: an experimental study with the common blackbird *Turdus merula*. J. Avian Biol. 46, 369–377. https://doi.org/10.1111/jav.00571

Sørensen, J.G., Kristensen, T.N., Loeschcke, V., 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6, 1025–1037. https://doi.org/10.1046/J.1461-0248.2003.00528.X

Wingfield, J.C., Manney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological bases of hormone-behavior interactions: the "emergency life history stage." Am. Zool. 38, 191–206. https://doi.org/10.1093/icb/38.1.191

Wingfield, J.C., Romero, L.M., 2015. Tempests, poxes, predators, and people: stress in wild animals and how they cope. Oxford University Press. https://doi.org/10.1093/ACPROF:OSO/9780195366693.001.0001

Figures

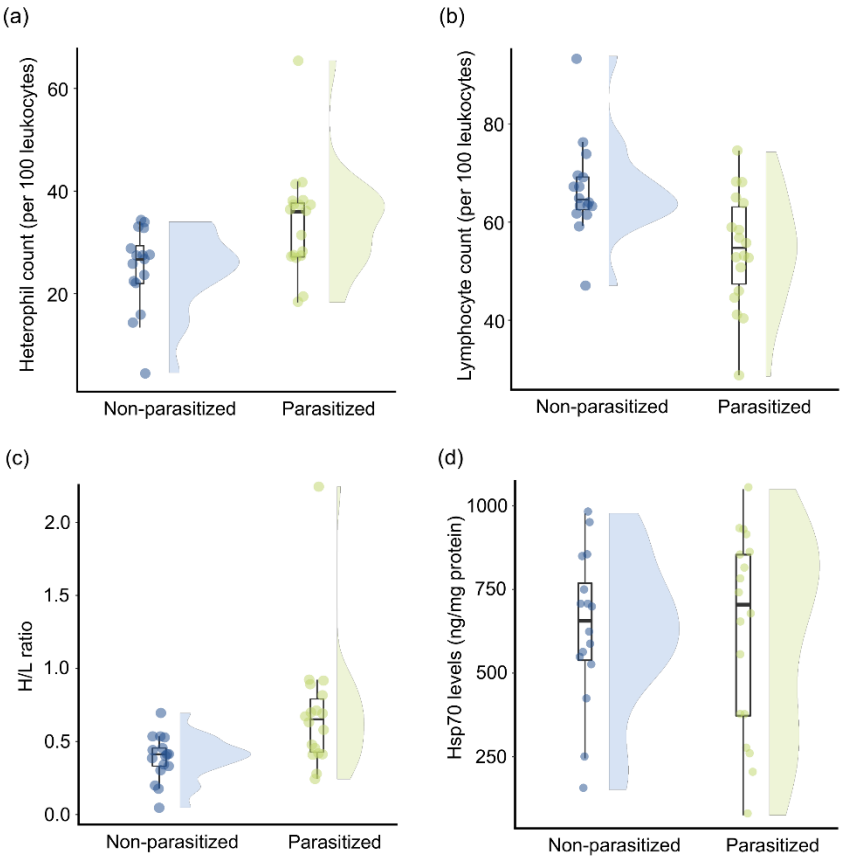

Figure 1. Differences in **(a)** heterophils, **(b)** lymphocites, **(c)** heterophils/lymphocites ratio (H/L ratio) and **(d)** heat-shock protein Hsp70 levels between parasitized and non-parastized control females. Boxplots show the median (bold line), and 25th and 75th percentiles (coloured boxes), with whiskers denoting the 5th and 95th percentiles. The violin plot outlines illustrate the probability density of data, i.e. the width of the shaded area indicates the proportion of the data located there.

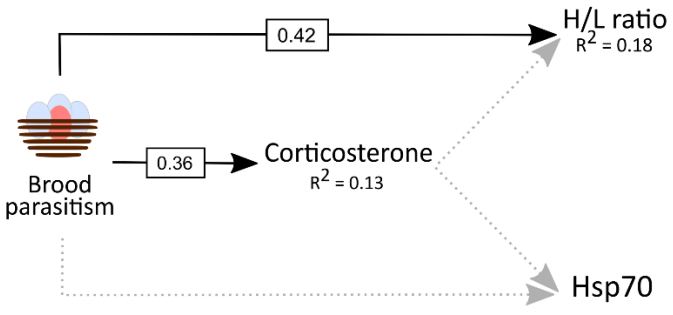

Figure 2. Structural equation model assessing for direct and indirect paths between experimental brood parasitism and the H/L ratio and Hsp70 levels. Plasma corticosterone concentration was included as an indirect path. Grey dotted arrows represent those paths that were tested in the full model but not included in the final model, of which paths are indicated by black arrows. Standardized effects are provided for those paths included in the final model.