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Hosts eject conspecific parasitic eggs according to the egg size in a passerine

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Conspecific brood parasitism (CBP) is an alternative reproductive tactic in egg-laying species, where a female lays her eggs in the nest of a conspecific. In a population of spotless starlings (*Sturnus unicolor*), we observed that some eggs are ejected from the nest during CBP events. It is unclear whether this ejection is a parasitic strategy (a host egg is ejected) or an anti-parasitism strategy (the parasitic egg is ejected). To clarify this, we genotyped the eggs ejected on the ground and found that 100% of them were parasitic. Egg discrimination might be based on tactile or visual cues, and we hypothesized that egg size could be used by hosts to eject parasitic eggs. We conducted experiments in the field using dummy eggs of varying sizes. The results showed that starlings were more likely to eject eggs if they were smaller than their own eggs. In contrast, no significant pattern of egg ejection was observed for larger eggs. Our results suggest that starlings use egg volume recognition as an anti-parasitism strategy to avoid the costs of parasitism. Whether this is a frequency-dependent strategy is worth further studies.

1. Introduction

Conspecific brood parasitism (CBP) is an alternative reproductive tactic found in egg-laying species in which a female lays her eggs in nests of conspecifics [1]. While it allows the parasite to reduce costs of the current reproduction—incubation and feeding nestlings—it increases those costs for the host [2]. One might expect the host to have evolved recognition and defence strategies towards foreign eggs, such as ejecting them from the nest [3–7]. Anti-parasitism behaviour usually requires the recognition, or at least the discrimination [8], of the parasitic egg. If recognition mechanisms are not precise enough, hosts could eject their own eggs. To discriminate, individuals might use tactile or visual cues [9]. In a conspecific system, host and parasite eggs are similar, making the recognition of foreign eggs by hosts challenging. While several studies have dealt with the mechanisms of egg ejection in heterospecific systems [9–14], to our knowledge, few studies have investigated such mechanisms in conspecific systems [15,16]. Understanding conspecific discrimination between own and foreign eggs remains an open question.

In starlings, several studies have found no egg ejection after clutch initiation [17–19], implying that once the egg-laying starts, hosts cannot discriminate between the eggs. However, eggs are often found on the ground (personal observation), and it is not clear whether egg ejection is a parasitism strategy (where a host egg is ejected by the parasite before laying [20–22]) or an anti-parasitism strategy (where the parasitic egg is ejected [18]). In the latter, hosts might have to discriminate the parasitic egg. According to the discordancy hypothesis [23], simple rules could be used to detect the foreign

egg, such as relative size differences between the parasitic and host eggs [9,24–26]. Indeed, parasitic eggs often differ in size from the host eggs [27].

We performed a study in the spotless starlings (*Sturnus unicolor*), a species where CBP is common (up to 37% of parasitized nests: [27,28]) and where egg size is repeatable for individual females [2,29]. As females lay one egg per day [30,31], we detected parasitism events by the appearance of two new eggs on the same day. Among those parasitic events, we have regularly observed one of the new eggs on the ground below the nest. The purpose of this study was first to genetically identify the host or parasitic origin of the eggs found on the ground and second to test whether hosts recognize parasitic eggs and eject them according to their size. We hypothesized that eggs on the ground resulted from an anti-parasitism strategy and that one of the cues used is based on the size difference between the eggs.

2. Methods

(a) Study site and population

This study was conducted during the reproductive season in 2022 and 2023 in a nest box colony of spotless starlings in central Spain (40°45'06.64" N; 3°48'41.09"W).

Adult starlings were captured at dawn from 28 February to 13 April 2022 and from 3 March to 13 April 2023, before the onset of laying, by closing the entrance of the nest box, and later by using spring traps. Birds were marked with an aluminium leg ring and a unique passive integrated transponder (PIT) tag inserted under the skin between the shoulder blades (Trovan Ltd, Douglas, UK). We collected a blood sample (kept in 0.5 ml 96% ethanol and stored at –20°C) from the brachial vein of all trapped birds for further DNA analyses.

Nest boxes were checked daily after detecting the first egg in the colony to follow the egg sequence and the occurrence of parasitism. Each new egg was marked with a non-toxic waterproof pen. Females lay one egg per day [30,31], thus the finding of two new eggs on the same day was recorded as a parasitism event. Occasionally, we found a new egg in the nest and a new egg on the ground below the nest.

PIT tag detections and pre-laying trapping data were used to identify the breeding female of each nest at each breeding attempt. For that, we placed an RFID antenna at the entrance of the nest (circular loop of wire the bird had to pass through), a battery and a PIT-tag reader (LID-650 decoder, Trovan Ltd, UK) for several days before laying, during incubation and nestling rearing. Moreover, we considered that the owner of the nest box was the female incubating at night, which was determined by checking all the nests with a long-range transponder reader (GR250, Trovan Ltd).

(b) Eggs on the ground

Eggs found on the ground below a box (often broken) during egg laying were collected in a leak-proof screw cap security container without touching it to prevent any contamination ($n = 15$). The inner shell membrane was separated from the shell with sterile pliers and stored in absolute ethanol at –20°C.

To discern the host or parasitic origin of the eggs found on the ground, we compared the genotypes from the inner shell membrane to the host female's genotype. The inner shell membrane, before embryo development, is composed of maternal DNA [32,33]. All eggs used were less than 24 h old; thus, we ensured that the genotyped DNA was maternal.

We extracted DNA from blood samples of females using a standard 10% Chelex protocol (Chelex[®] 100 Resin, Bio-Rad), and from egg membranes ($n = 15$), using the Qiagen[®] DNeasy Blood & Tissue Kit (Qiagen, France). The membranes were cut into small pieces with sterile scissors, and the lysis was held overnight at 56°C. Subsequent steps were performed according to the manufacturer's instructions. Genotypes were obtained for four polymorphic microsatellite loci: Sta269 [34], and three new loci produced by AllGenetics (Spain): Sta212, Sta338 and Sta360 (table 1). Each locus was amplified in simplex PCRs in a reaction of 15 μ l with 1 \times Tp5x (5 \times Colorless GoTaq[®] Flexi Buffer, Promega), 1.5 mM MgCl₂ (Promega, USA), 0.5 mM of each dNTP, 1 μ M of each forward and reverse primer, 1 U GoTaq G2 Flexi polymerase (Promega), 2.65 μ l RNase-free water and 3.8 μ l of DNA sample. PCRs were done on a TProfessional Basic 96 thermocycler (Biometra, Germany) following the cycling conditions described in [34]. The number of alleles per locus, expected (H_e) and observed (H_o) heterozygosities, and the proportion of null alleles for each locus were calculated using CERVUS 3.0.7 [35] (table 1). Genotyping was performed using an Applied Biosystems 3730XL DNA Analyzer at the genomic platform of Genoscreen (Lille, France). Fragment lengths were manually checked on chromatograms to detect inconsistencies, and genotypes were scored against the GeneScan-500 Liz Size Standard (Applied Biosystems) using Peak Scanner Software v. 1.0 (Applied BiosystemsTM).

We compared the genotypes of the egg on the ground to the genotypes of the host female of the corresponding nest. If the egg on the ground had been laid by the host female, the DNA found in its membrane would be identical to that of the female. Consequently, if the genotypes were different at least at one locus, the egg on the ground would have been laid by another female, i.e. a parasite.

(c) Egg ejection experiment

We simulated 63 parasitism events by adding one dummy egg (three-dimensional-printed plastic or plaster eggs) during first and replacement clutches in 2023. To print the plastic eggs, we used an in-built model available within the three-dimensional

Table 1. Variability at the four microsatellite loci amplified for host female spotless starlings of the population. T_a , annealing temperature; H_o , observed heterozygosity; H_e , expected heterozygosity; HW, Hardy–Weinberg equilibrium test significance; F (Null), estimated frequency of null alleles. Apart from the blood samples of the 14 host females, we genotyped 28 other females, selected at random from the population, to calculate H_e .

locus	repeat motif	primer sequence	T_a (°C)	no. of alleles	product size range	H_e	H_o	HW	F (Null)
Sta269	(CA) ₁₅	TGGGGATTAATAGGGGTGTG GCAGTGAGAAGAGGGCTTTG	58	14	181–211	0.837	0.767	NS	0.041
Sta212	(AATG) ₁₁	GATCTCGGACAGCAGTCTC CCACTGCAGACATCAGGGTT	60	5	230–250	0.777	0.452	++	0.256
Sta338	(AC) ₁₀	CTGACTTGCTTGTAGTCCCA AGCTGCAGACACAACCAAGA	60	8	98–114	0.724	0.674	NS	0.040
Sta360	(ATCCC) ₆	TGTCAGCAACTGCAGTGAGA TATGCTGTCTGCTCCATGC	60	13	267–292	0.873	0.805	NS	0.037

Table 2. Description of 2051 eggs measured in 2019; and length, width and volume of three-dimensional printed eggs. Lengths and widths of dummy eggs were calculated by the addition or subtraction of 1 or 2 s.d. from the mean values of 2019.

		length (mm)	width (mm)	volume (cm ³)
natural eggs	minimum	21.14	18.64	4.50
	maximum	34.34	31.39	14.01
	mean	29.96	21.48	7.07
	s.d.	1.39	0.83	0.72
dummy eggs	control – 2 s.d.	27.19	19.82	5.45
	control – 1 s.d.	28.58	20.65	6.22
	control	29.96	21.48	7.05
	control + 1 s.d.	31.35	22.31	7.96
	control + 2 s.d.	32.74	23.14	8.94

Table 3. Sample size (N) and range of differences between the dummy egg volume and the mean volume of host eggs for each experimental category.

category	N	volume difference (cm ³)
–2 s.d.	9	[–2.29 : –1.63]
–1 s.d.	11	[–1.44 : –0.90]
Control	25	[–0.57 : 0.46]
+1 s.d.	10	[0.77 : 1.70]
+2 s.d.	8	[1.91 : 2.54]

printer software, and we modified the length, width and weight to match the shape of starling eggs. Using data from egg measurements collected in 2019 ($n = 2051$), we calculated the minimum, maximum and average volume of eggs in the population. We printed plastic eggs within the natural range of the population (table 2). According to our dataset, a difference of one standard deviation (s.d.) from the mean volume corresponds to 1 cm³. We printed five categories: –2 cm³, –1 cm³, control, +1 cm³ and +2 cm³—using the mean volume in the population as our control volume (electronic supplementary material, figure A1). After printing, the eggs were sanded and painted using an airbrush (Shenzhen Tongshi Technology, China), and three colours: white (Tamiya Colour—Acrylic Paint XF-2), sky blue (X-14) and light blue (XF-23) in a 10 : 1 : 1 ratio to reproduce starling eggs' colour. Plaster eggs were made following Soler *et al.* [36]. To make sure the effect of dummy egg addition, if any, was not due to the egg material, we compared the ejection rate between natural ($n = 74$), plaster ($n = 14$) and plastic eggs ($n = 49$). For the natural type, we used natural parasitism in the population and quantified the percentage of egg ejection. As most of the ejected eggs were broken, we do not have data on their size. All nests were checked daily and suffered similar experimental disturbances.

Most natural parasitism events occur on day 2 or 3 of the laying sequence (personal observation). To reproduce natural parasitism, we placed a dummy egg (in plastic, $n = 49$ or in plaster, $n = 14$) in 54 nests on day 3 of laying (nine nests were used in two different clutches). We used only nests without signs of previous parasitism and with a regular laying pattern, i.e. nests with three natural eggs on day 3. We measured the length and the width of these three eggs with digital callipers (Mitutoyo, Japan) to the nearest 0.01 mm. Egg volume was calculated by the formula: $0.51 \times \text{length} \times \text{width}^2$ [37]. Nests were randomly assigned to each category. We selected the dummy egg that most closely matched the target volume defined by the assigned category (see table 3 for details).

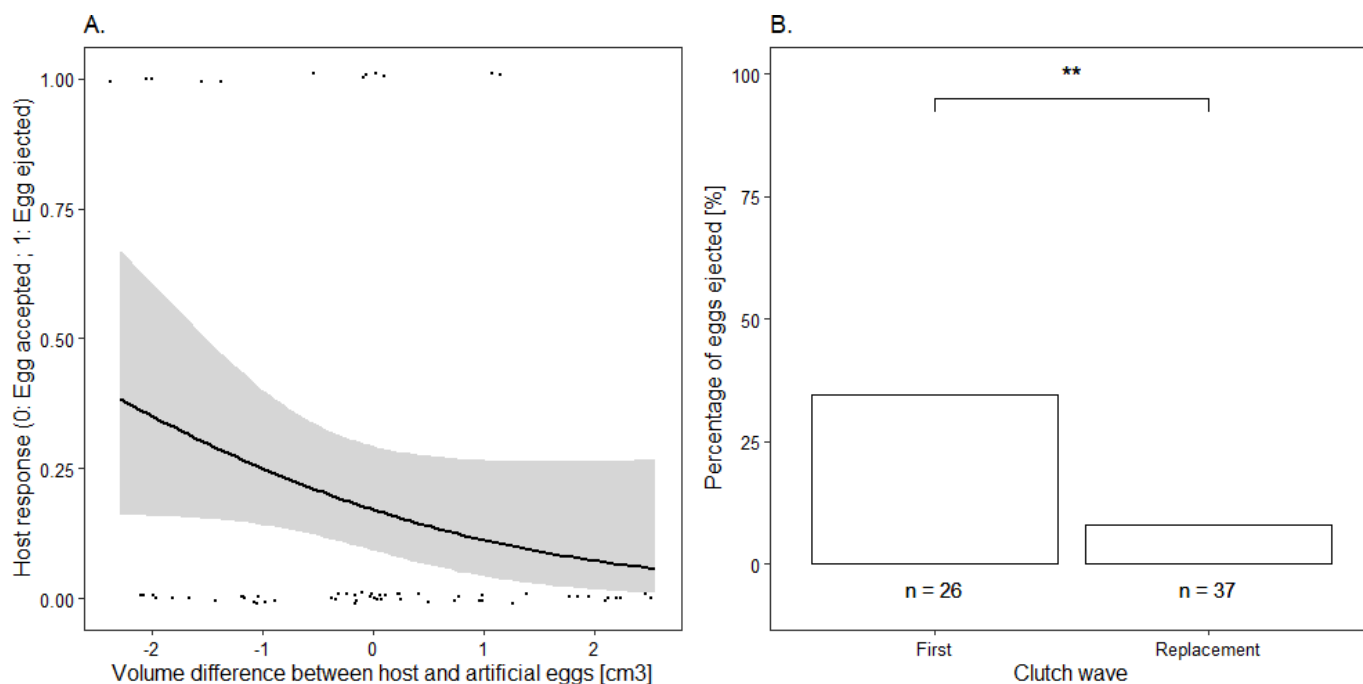


Figure 1. Relationship between the host response (accept or eject eggs) and the relative volume difference between the dummy and the three host eggs (A) and percentage of ejected eggs per clutch wave (B). Dots represent the actual data; the line shows predicted values. The grey area around the line denotes the 95% confidence interval. See text for statistics.

We followed nests daily to detect ejection behaviour. If the dummy egg was not in the nest box, we considered it ‘ejected’. On the contrary, if it was still inside the nest box 3 days after the inclusion of the dummy egg, we considered it ‘accepted’ and removed it from the nest box. Nest desertion is common in brood parasitism [16]; we also monitored this response.

(d) Data analysis

To compare ejection rates between plastic, natural or plaster eggs, we performed a Kruskal–Wallis test using the R package *coin* [38].

To study whether the acceptance of dummy eggs was related to the host’s mean egg volume, we applied a generalized linear mixed-effects model (GLMM) for binomial data with the response (accepted or ejected) as the dependent variable using the R package *lme4* [39]. We included as fixed effects the relative difference in volume (volume of the dummy egg minus mean volume of the host eggs) as a continuous variable and the clutch wave (first or replacement clutch) as a factor. We included the nest as a random factor.

Alternatively, we tested whether ejection was related to just the length or width of the egg. Thus, we applied a similar GLMM for binomial data, but as fixed effects, we included the relative difference in length and width between the dummy egg and the host eggs as continuous variables. We included the nest as a random factor.

All continuous variables were standardized using the function *scale*. Nonlinear relationships were tested, but as they were non-significant, they were removed from the final models.

3. Results

(a) Eggs on the ground

During 2022 and 2023, we found 15 parasitism events in which one of the new eggs was on the ground. These eggs on the ground were found on day 1 ($n = 4$), 2 ($n = 4$), 3 ($n = 6$) or 4 ($n = 1$) of the host laying sequence (modal clutch size is five eggs [40]). They stemmed from 14 nests with known host females (one nest was parasitized twice). The analysis of the 15 egg membranes revealed the presence of DNA in 100% of the samples. Negative PCR controls confirmed the absence of contamination for all loci. All samples showed one (homozygous) or two (heterozygous) alleles per locus (electronic supplementary material, table S1). For two eggs, we failed to amplify DNA at one locus; thus, we only had three loci for those eggs. For 13 eggs, the genotypes of the egg membrane and the host female were different at all the loci (electronic supplementary material, table S1). For two eggs, genotypes did not match at three and two loci, respectively. All the eggs found on the ground and analysed exhibited DNA profiles that differed from those of the corresponding host females.

(b) Eggs ejected

No nest was deserted during our experiment. In total, 20% of the eggs were ejected (22% of the natural eggs: 16 out of 74; 14% of the plaster eggs: two out of 14; and 20% of the plastic eggs: 10 out of 49). Most of the dummy eggs disappeared. The proportion

of eggs ejected by hosts was not significantly different between the three materials (natural, plaster, plastic) (Kruskal–Wallis: $X^2_2 = 0.387$, $p = 0.868$).

We found that smaller eggs relative to the host eggs were significantly more often ejected than the other eggs. Eggs smaller than host eggs by 2 s.d. were 10.9 times more likely to be ejected than other eggs (GLMM for binomial data: $X^2_1 = 4.879$, $\beta = -1.193 \pm 0.540$, $p = 0.027$, $R^2 = 0.215$, $n = 63$; figure 1A). Starlings ejected eggs that were at least 0.52 s.d. [CI = -1.601–0.515] smaller than theirs (7% smaller), and did not eject eggs that were larger. Moreover, hosts ejected significantly more eggs during the first than during the replacement clutch wave ($X^2_1 = 7426$, $\beta = -2.506 \pm 0.920$, $p = 0.006$, $R^2 = 0.258$, $n = 63$; figure 1B).

We found no effect of the length nor the width relative differences on the host response (GLMM for binomial data: $p = 0.958$ and $p = 0.190$ for the relative difference in length and width, respectively).

4. Discussion

Our results suggest that spotless starlings removed parasitic eggs from their nest during the egg-laying period, and they used their relative size to detect them. The former result contrasts with several studies that have observed ejection before laying but not after clutch initiation in starlings [5,18,22]. However, other passerines do eject after clutch initiation [16,41]. Moreover, egg disappearance during the dummy egg experiment suggests that more natural eggs than detected may have been naturally removed from nests. A study on American robins (*Turdus migratorius*) showed that egg ejection distances ranged from 0 to 57.6 m [42]. Other studies on starlings have proposed (though not genetically tested) that parasite females were responsible for eggs ejected once the hosts initiated their clutch [20,21,27]. However, none of our analysed eggs matched the DNA profile of the host female, supporting the idea that the eggs removed were parasitic. Whereas in our study we do not know whether males and females ejected parasitic eggs, Pinxten *et al.* [18] showed that male resident European starlings rejected dummy eggs when they were the first bird of the couple to visit the nest after egg addition, and Eens & Pinxten [17] showed that both hosts removed eggs added before clutch completion in spotless starlings.

Our results show that eggs were more likely to be ejected when they were significantly smaller than the host eggs. Rothstein [23] differentiated two mechanisms of egg recognition. The first mechanism is true egg recognition, which occurs when individuals can recognize foreign eggs no matter how many there are. Conversely, individuals proceed with the recognition by discordancy when they reject the most different egg in a clutch. In our protocol, we introduced a unique dummy egg into a clutch of three eggs, thus creating a situation where only one egg was discordant within the clutch (except in the control condition). Hosts significantly ejected smaller eggs (compared to their own), which supports the hypothesis of recognition by discordancy. However, our experiment does not allow us to discard the hypothesis of true recognition. The selective ejection of only the smallest eggs, and not the largest, can be explained by several potential factors. The ejection of parasitic eggs might be limited by the hosts' size—bigger eggs are more difficult to reject than smaller ones [43–45]. Egg size and female size are two correlated traits [2], and it would be worth testing whether beak width could be a limiting factor of egg ejection. Alternatively, according to the parental investment theory, bigger eggs carry higher fitness prospects than smaller eggs [46] and are usually preferred by parents [47]. Therefore, ejecting one's own large egg by mistake would be more costly than ejecting a small one [9]. In any case, the significant ejection of the smallest eggs (approx. 20% of our dummy eggs) during our experiment is similar to what is observed in natural cases (22% of natural parasitic eggs ejected). Finally, our results indicate that hosts ejected dummy eggs, especially during first rather than replacement clutches. A replacement clutch can be laid by females after losing their initial clutch [48], or by females taking over a nest. The former may be particularly cautious to avoid the potential error cost of ejecting one of their eggs instead of the parasites [16], and the latter are usually young and inexperienced females (personal observation) who might still have to hone their discrimination abilities.

Nevertheless, we find multiple cases of successful parasitism in our colony every year [28], showing that some hosts raise parasitic eggs. Either some hosts fail to differentiate the eggs and do not eject the parasitic eggs, or else they discriminate but accept the stranger egg. According to Hamilton's rule [49], altruistic behaviour can evolve if the genetic relatedness between the altruist and the recipient makes the benefits outweigh the costs. In the case of kinship between females (host and parasite), the host could show altruism towards the parasitic egg and raise it. To determine whether they fail to recognize the eggs or recognize them but do not discriminate against them, more experiments are needed.

In conclusion, we describe for the first time egg ejection behaviour by hosts in starlings, which fits the expectations of an anti-parasitism strategy. To deal with CBP, some spotless starlings eject parasitic eggs from their nests. Additionally, hosts recognize dummy eggs based on size discrepancies within the clutch, suggesting that spotless starlings rely on size discrepancy to discriminate parasitic eggs. Under field conditions, it is difficult to detect those ejected eggs, and we suspect that it is probably a more common strategy than previously assumed. Parasitism is costly for the host, and the rates in our study population are high (between 13% and 25% of reproductive events are parasitized every year, personal observation), which could have selected basic recognition mechanisms to lower the fitness consequences. As other studies have shown no egg ejection after clutch completion in starlings, it could be a population-specific behaviour. Additional studies would be needed to determine if spotless starlings of other colonies exhibit the same anti-parasitism behaviour.

Ethics. All applicable institutional and national guidelines for the care and use of animals were followed. Permission to work in the area and capture birds was granted by permits from the Consejería de Medio Ambiente (Comunidad de Madrid, Spain) (ref. AUT.068.22; AUT.041.23; 10/261763.9/23; 10/090960.9/23) and the city council of Soto del Real.

Data accessibility. The data description and code are available from the Dryad Digital Repository [50].

Supplementary material is available online [51].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. A.L.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, visualization, writing—original draft; D.G.: investigation, project administration, writing—review and editing; C.P.: conceptualization, formal analysis, methodology, resources, writing—review and editing; R.M.: conceptualization, data curation, investigation, methodology, project administration, resources, supervision, validation, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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