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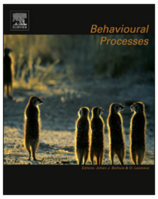

Female and male rufous horneros eject shiny cowbird eggs using a mental template of the size of their own eggs

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ABSTRACT

Hosts of interspecific brood parasites often evolve antiparasitic defences, like the recognition and rejection of parasite's eggs. Most hosts use differences in coloration and maculation to discriminate between their own and parasitic eggs, but there are a few cases of hosts using the size of eggs as a cue. To recognize parasite eggs, hosts may require the presence of their own eggs and use a discordancy rule or may use a mental template of their own eggs. Females are responsible for egg rejection in hosts in which they incubate alone, but if incubation is shared, males can also reject parasitic eggs. The rufous hornero, *Furnarius rufus*, a host of the shiny cowbird *Molothrus bonariensis*, ejects parasite eggs using egg size as a cue. We studied the cognitive mechanism underlying the recognition and ejection of parasitic eggs by this host. We experimentally parasitized hornero nests with eggs of different size, with and without the presence of host eggs and determined which sex was responsible for the ejection. We found that horneros ejected parasitic eggs using the size of the egg as a cue and did not need to compare parasitic eggs with their own eggs, which is consistent with the hypothesis of a mental template. Females and males ejected eggs at similar frequencies. We also found that cowbird eggs laid in hornero nests were longer and wider than those laid in nests of other host in the same area, which is consistent with the hypothesis of host-specific female cowbird lineages evolving larger eggs to deceit horneros from recognizing and ejecting their eggs.

1. Introduction

Interspecific brood parasites exploit the parental care from individuals of other species, the hosts, which incubate the parasite egg and feed the parasite young. This exploitation entails fitness costs for the hosts that may select for the evolution of host antiparasitic defences, which in turn, may select for parasite's counterdefenses resulting in a coevolutionary arms race (Rothstein, 1990; Davies, 2011; Soler, 2017). The most common antiparasitic defence is the rejection of parasite eggs (Soler, 2014) either by ejection (Davies and Brooke, 1988; Sealy and Neudorf, 1995; Servedio and Hauber, 2006; Grim et al., 2014), burial (Moskát and Honza, 2002; Guigueno and Sealy, 2010) or nest desertion (Goguen and Mathews, 1996; Hosoi and Rothstein, 2000). The majority of hosts use differences in eggshell characteristics (i.e. background coloration and maculation) between their own and parasite eggs to recognize and reject foreign eggs (Krüger, 2007; Polačikova et al., 2007; Abolins-Abols et al., 2019), while it is less common for hosts to use differences in egg size or shape as a cue (but see Mason and Rothstein, 1986; Alvarez, 2000; Marchetti, 2000; Segura et al., 2016). Hosts may recognize the parasite egg by comparing its appearance when host and parasite eggs are simultaneously in the nest and eject the egg that is different from the majority (recognition by discordancy; Rothstein, 1974, 1975a; Moskát et al., 2010, 2014). Alternatively, hosts may always be able to recognize a foreign egg by comparing it with a template or mental image that can be innate or learned (true egg recognition; Rothstein, 1974, 1975a; Hauber and Sherman, 2001; Moskát et al., 2010) although in some cases these hypotheses are not mutually exclusive (Yang et al., 2014; Segura et al., 2016). In species in which only females incubate, they are the sex responsible for egg rejection (Rohwer et al., 1989; Lotem et al., 1995; Palomino et al., 1998; Požgayová et al., 2009), while in species where incubation is shared, both sexes may reject the parasite egg (Soler et al., 2002; Lee et al., 2005; Honza et al., 2007).

The shiny cowbird, *Molothrus bonariensis*, is an extreme generalist brood parasite whose eggs have been found in nests of over 260 species (Lowther, 2018). Shiny cowbirds reduce the reproductive success of

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their hosts (Massoni and Reboreda, 1998; Astié and Reboreda, 2006; Tuero et al., 2007) and in many hosts these costs have selected for the evolution of antiparasitic defences (Reboreda et al., 2013). The rufous hornero, *Furnarius rufus*, is a host of the shiny cowbird (Fraga, 1980; Mason, 1986), it is sympatric with this parasite across all its distribution (Ortega, 1998; Remsen, 2003) and it ejects cowbird eggs by grasping them at the widest part using as a cue differences in size between their own and parasite eggs (Mason and Rothstein, 1986). Horneros' use of egg size, instead of colour or maculation, as a cue to discriminate and eject cowbird eggs would be an adaptation to recognize foreign eggs in the darkened interior of the incubation chamber of their closed "oven" nests (Mason and Rothstein, 1986).

The rufous hornero-shiny cowbird system provides a singular model to explore the cognitive mechanism underlying the recognition and ejection of parasitic eggs in a host using egg size to discriminate between their own and parasitic eggs. Besides, in this host parental care is highly egalitarian between sexes (Massoni et al., 2012), with males sharing incubation with females, and therefore it would be expected that males also eject parasite eggs. In this paper we addressed two main questions: 1) whether rufus horneros eject shiny cowbird eggs using a discordancy rule or they have template-based recognition, and 2) the sex responsible for the ejection of parasite's eggs. Also, we analysed if this antiparasitic defence have selected for an increase in egg size in shiny cowbirds that parasitize rufus horneros compared to those that parasitize other host in the same area.

2. Methods

2.1. Study area

The study was conducted at “Instituto Nacional de Investigación Agropecuaria Las Brujas Wilson Ferreira Aldunate” (INIA), in the department of Canelones, Uruguay (34°40′14″ S, 56°20′27″ W). The study site is an agroecosystem of 451 ha. Rufous horneros (hereafter horneros) and shiny cowbirds (hereafter cowbirds) are common and all year residents. Other common hosts of cowbirds present in the study site are chalk-browed mockingbirds, *Mimus saturninus*, rufous-bellied thrushes, *Turdus rufiventris*, creamy-bellied thrushes, *Turdus amaurochalinus*, and rufous-collared sparrows, *Zonotrichia capensis*.

2.2. Data collection and analysis

We searched for hornero nests within the study area throughout the breeding season (late August-late November). We limited data collection to nests built at a height of up to 4 m, which could be reached with a portable ladder. We monitored 93 hornero nests, 53 found before laying, 13 during laying, and 27 during incubation.

Horneros build a conspicuously exposed closed “oven” nest consisting of mud mixed with grass or dung. The nest is divided in an entrance and an egg or incubation chamber, its wall is between 3 and 5 cm thick and it may weigh up to 5 kg (Remsen, 2003). To inspect the nest for the first time, we made a hole with a circular saw in the wall of the incubation chamber. Then we closed the hole with a circular wooden plug and covered it with fresh mud. In subsequent visits we removed the plug and then closed the hole again (Fraga, 1980). In each visit, we recorded the nest contents and marked new eggs with waterproof ink and measured their length and width with a calliper (± 0.1 mm). Hornero and cowbird eggs are easily distinguishable by size (see results), shape (parasite eggs are more rounded than host eggs) and eggshell colour and maculation (most parasite eggs are spotted with background shell colour pure white, light cream, light bluish white, light greenish white, dark cream, or light brown, while host eggs are immaculate white).

We also found nests of other frequently reported cowbird hosts that nested in the study site: chalked-browed mockingbirds ($n = 42$), rufous-bellied thrushes ($n = 17$), creamy-bellied thrushes ($n = 11$) and rufous-collared sparrows ($n = 13$).

We estimated the frequency of parasitism as the proportion of nests with cowbird eggs and intensity of parasitism as the average number of parasitic eggs in parasitized nests. For these estimations we used a sample of hornero nests found during construction and laying that completed laying ($n = 34$). We considered that the clutch was complete when the nest presented a constant number of eggs for at least two consecutive visits. For the other hosts we considered all nests because the small sample size.

2.3. Artificial parasitism experiments

We used model eggs made of plaster-of-Paris and painted with white acrylic paint as a previous study showed that experiments with artificial eggs yield similar results that those with real eggs (Mason and Rothstein, 1986). Model eggs were made using latex moulds build using real cowbird and hornero eggs. We used two types of cowbird model eggs: small (22.3 ± 0.08 × 17.4 ± 0.1 mm, mean ± sem, $n = 30$) and large (24.3 ± 0.08 × 20.3 ± 0.07 mm, $n = 10$) and one control hornero model eggs (27.6 ± 0.09 × 21.0 ± 0.12 mm, $n = 10$). To parasitize nests, we removed the plug in the wall of the incubation chamber, introduced the artificial egg and then closed the hole again (Fraga, 1980). We conducted the experiments in the morning during laying and early incubation. Nests were checked for ejection every 1–2 days. We considered that a parasitic egg was ejected if it disappeared from the nest and accepted if it remained in the nest for at least five days after the experimental introduction (Rothstein, 1975b), except for treatment 4 (see below). Horneros do not puncture eject parasite eggs, instead they eject eggs by grasping them at the widest part (Mason and Rothstein, 1986, supplementary videos). Consistent with this, in several cases we found intact natural cowbird eggs that had been rejected at the base of hornero nests.

We conducted four experimental treatments by adding: 1) one small cowbird egg ($n = 10$ nests), 2) one large cowbird egg ($n = 10$ nests), 3) one hornero egg ($n = 10$ nests), and 4) two small cowbird eggs ($n = 10$ nests). In the first three treatments no host eggs were removed in conjunction with experimental parasitism. We added artificial eggs to clutches containing 2–4 host eggs. Treatment 4 took place the day the host laid its first egg, which was removed and substituted by two small cowbird eggs. Therefore, in this treatment horneros were unable to compare cowbird eggs with their own eggs. Treatment 4 lasted only 1 day because the following day the female hornero laid another egg, which would allow nest owners to compare the cowbird with their own egg. For each experimental nest we determined the relative length and width of the artificial egg as the length or width of the artificial egg over the average length or width of the clutch of the experimental nest. In treatment 4 we averaged the relative length and width of the two small cowbird eggs used for the experiment.

2.4. Timing and sex involved in egg rejection

We captured horneros using a small circular net placed at the nest entrance when the birds were inside. We took some morphological measurements (body mass, bill length, bill breath) and then we took a small blood sample (15–30 μl) through brachial vein puncture with a 31 G needle. We collected the blood with an 60 μl heparinized capillary tube, immediately mixed it with 0.5 mL of lysis buffer and stored it at room temperature until analysis. Before release, each bird was banded with a numbered metal ring and a unique colour combination of plastic rings. We sexed individuals via polymerase chain reaction (PCR) amplification of the CHD-Z and CHD-W alleles using the P2/P8 primer pair (Griffiths et al., 1998).

To determine the sex of the individual that ejected the cowbird egg and the timing of ejection we placed a video recorder (Sony Handycam DCR-HC28) 10 m away from the nest. Recordings started once the small model cowbird eggs (those used in treatments 1 and 4) were placed in the nest, and lasted for 4 − 6 h. We analysed the following variables: 1) sex of the individual that ejected the egg, 2) time elapsed between experimental parasitism and the first visit to the nest by the individual that ejected the egg, 3) time elapsed between the first visit by the individual that ejected the egg and egg ejection, 4) number visits to the nest done by the individual that ejected the egg before ejection, and 5) time spent inside the nest by the individual that ejected the egg. In the cases at which 2 small cowbird eggs were introduced in the nest, the reported data are for the first ejected egg only.

2.5. Statistical analysis

To compare the egg size of horneros with that of cowbird eggs in hornero nests we averaged the length and width of the hornero eggs of the same clutch as we consider this variable would be more appropriate if horneros have a template or mental image of their own eggs. We analysed differences in size between hornero eggs and cowbird eggs laid in their nests using Wilcoxon tests, while we analysed differences in size between cowbird eggs laid in hornero nests and those laid in mockingbird nests using Mann-Whitney *U* tests. To compare the proportion of ejected eggs in experiments of artificial parasitism we used Fisher's exact tests and to compare differences between sexes in timing of ejection we used Mann-Whitney *U* tests. Statistical tests were performed using R (R Development Core Team 2015) with $P < 0.05$ (two-tailed). We presented data as means ± mean standard errors.

2.6. Ethical statement

Field data collection and experimental procedures were approved by the ethical committee of the Universidad de la República, Uruguay (Comisión de Etica en el Uso de Animales, Facultad de Ciencias, UdelaR, 241000-001186-12).

3. Results

3.1. Natural parasitism

At our study site, the hornero was a common host of the cowbird. The frequency of parasitism was 44.2 % (n = 34 nests found during construction and laying that completed laying) and the intensity of parasitism was 1.7 ± 1.1 eggs/nest (n = 15 parasitized nests, range 1–5 cowbird eggs per nest). Frequencies and intensities of parasitism in other hosts in the study area were: chalk-browed mockingbirds 70.3 % (n = 42 nests) and 1.2 ± 0.4 eggs/nest (n = 30 parasitized nests), rufous-bellied thrushes 29.4 % (n = 17 nests) and 1.4 ± 0.9 eggs/nest (n = 5 parasitized nests), creamy-bellied thrushes 27.3 % (n = 11 nests) and 1.0 ± 0.0 eggs/nest (n = 3 parasitized nests) and rufous-collared sparrows 7.7 % (n = 13 nests) and 2.0 ± 0.0 eggs/nest (n = 1 parasitized nest).

Hornero eggs were 28.1 ± 0.2 mm in length (range 25.7–31.0 mm) and 21.4 ± 0.1 mm in width (range 20.1–23.1 mm) (n = 49 nests, Fig. 1A, B). Cowbird eggs in hornero nests were 25.1 ± 0.14 in length (range 23.0–27.5 mm) and 20.5 ± 0.1 in width (range 18.6–21.7 mm) (n = 49 eggs) while cowbird eggs in mockingbird nests were 24.3 ± 0.19 in length (range 22.2–28.1 mm) and 20.0 ± 0.2 in width (range 16.5–22.5 mm) (n = 35 eggs) (Fig. 1A, B). Hornero eggs were longer and wider than cowbird eggs found in their nests (Wilcoxon tests; length, z = 6.0, $P < 0.001$; width, z = 5.3, $P < 0.001$), while cowbird eggs in hornero nests were longer and wider than those found in nests of mockingbirds (Mann Whitney *U* tests; length U = 458, $P < 0.001$; width: U = 552, $P < 0.01$) (Fig. 1A, B).

3.2. Artificial parasitism experiments

All rejections were by egg ejection (we did not observe any case of rejection by nest desertion). Fig. 2A shows the relative length and width of the eggs used in the experiments of artificial parasitism. Horneros ejected small cowbird eggs in 10/10 cases, large cowbird eggs in 4/10 cases and hornero eggs in 2/10 cases (Fig. 2B). The frequency of ejection of small cowbird eggs was higher than that of large cowbird eggs (Fisher's exact test $P < 0.05$) and control hornero eggs (Fisher's exact test $P < 0.001$). In the treatment with two small cowbird eggs both eggs were ejected in 9/10 cases and in the other case one egg was ejected and one remained in the nest for more than 24 h. The frequency of ejection of small cowbird eggs did not differ between treatments in which horneros could and could not compare the cowbird egg with their own eggs (Fisher's exact test P = 1). The relative width of the narrowest cowbird egg accepted was 0.89 while the relative length of the shortest cowbird egg accepted was 0.78. We also observed ejection of natural cowbird eggs in 7/30 parasitized hornero nests.

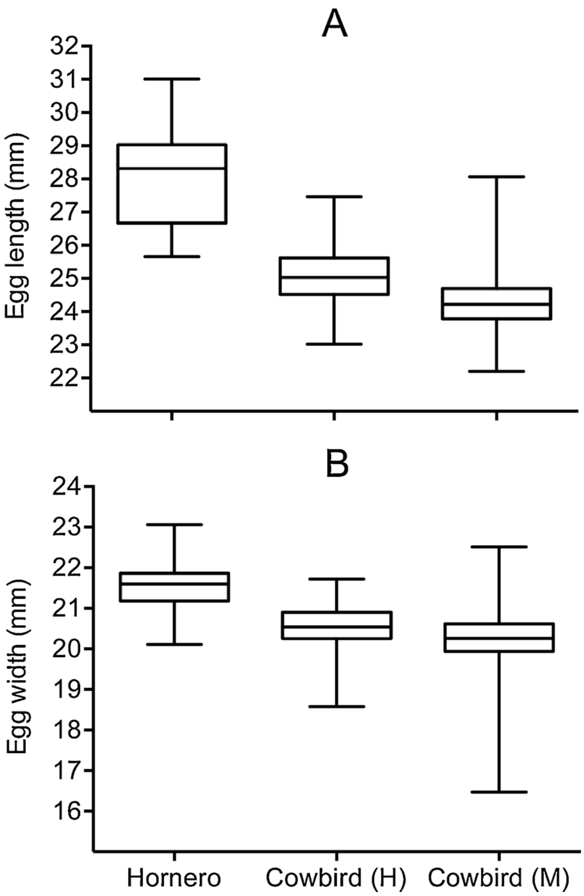

Fig. 1. Box plots indicate the length and width (median, lower and upper quartiles, and smallest and largest values) of rufous hornero (*Furnarius rufus*) and shiny cowbird (*Molothrus bonariensis*) eggs laid in nests of horneros (Cowbird H) or in nests of chalk-browed mockingbirds (*Mimus saturninus*) (Cowbird M). Sample sizes are: hornero eggs n = 49 clutches, cowbird eggs laid in hornero nests n = 49 eggs, cowbird eggs laid in mockingbird nests (n = 35 eggs).

3.3. Sex involved and timing of egg ejection

Females and males do not differ in bill length (females 25.0 ± 0.31 mm, n = 14, males 25.4 ± 0.4 mm, n = 12; Mann-Whitney U test, U = 110, P = 0.36) and width (females 4.5 ± 0.05, n = 14, males 4.56 ± 0.09, n = 12; Mann-Whitney U test, U = 97, P = 0.77) and therefore they have similar grasp indices (Rohwer and Spaw, 1988; Moksnes et al., 1991). We video recorded 12 events of egg ejection. All ejections were by grasping the model egg at the widest part. Females ejected the cowbird egg in 7 cases while males ejected the egg in the other 5 cases (Binomial test P = 0.30). The time elapsed since we

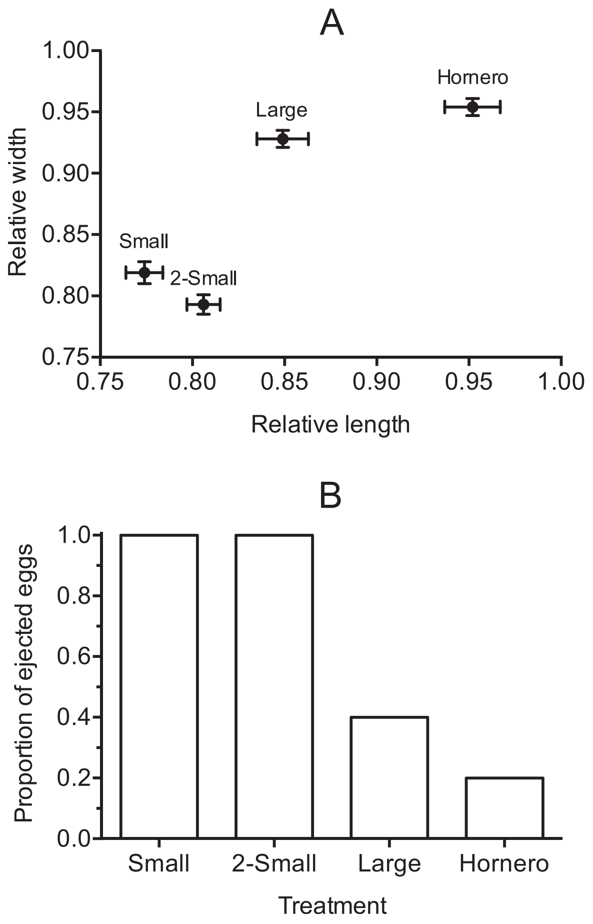

Fig. 2. Experiments of artificial parasitism in rufous hornero nests. (A) Relative length and width ± SE of the eggs used in the experiments of artificial parasitism (see details in methods). (B) Proportion of ejected eggs in the experiments of artificial parasitism. In the treatments "Small", "Large" and "Hornero" we added, respectively, one small cowbird egg, one large cowbird egg, or one hornero egg to a clutch containing 2-4 hornero eggs and these treatments lasted 5 days. In the treatment "2-Small" we added two small cowbird eggs after removing the first egg laid by the host and this treatment lasted one day. Sample size in each treatment was 10 nests.

Table 1
Ejection behaviour of small cowbird eggs by female and male horneros. Latency of the first visit after parasitism indicates the time elapsed between experimental parasitism and the first visit to the nest by the individual that ejected the egg, latency of ejection indicates the time elapsed between egg ejection and the first visit by the individual that ejected the egg and time in the nest before ejection indicates the time spent inside the nest by the individual that ejected the egg. Times are indicated in seconds (mean ± SE). Differences between sexes were analysed with Mann-Whitney *U* tests.

Variable	Females (n = 7)	Males (n = 5)	P
Latency of first visit after parasitism	873.3 ± 290.6	3004.0 ± 775.3	0.04
Latency of ejection	3881.7 ± 1612.2	4087.0 ± 1821.7	0.94
# of nest visits before ejection	4.9 ± 1.9	6.0 ± 2.6	0.57
Time in the nest before ejection	1891.7 ± 614.3	2148.6 ± 1077.6	0.94

parasitized the nest and the individual that ejected the egg visited the nest for the first time was shorter for females than males, but there were no sexual differences in the latency until egg ejection, the number of nest visits before ejection and the time spent in the nest before ejection (Table 1). In most cases (10/12) horneros ejected the model egg by grasping it with its beak and dropping it in the nest entrance tunnel without leaving the brood chamber. The egg remained in the entrance tunnel and the same individual dropped it onto the ground in a later visit (6/10 cases) or the egg rolled away and fell onto the ground (4/10 cases). Only in 2/12 cases the individual carried the egg in its beak while flying away directly from the brood chamber (Supporting Online Material).

4. Discussion

We showed that female and male horneros eject cowbird model eggs using egg size as a cue and they do not need to compare the parasite egg with their own eggs, which is consistent with the hypothesis of a template or mental image of their own eggs. Small cowbird model eggs were ejected more frequently than large cowbird model or control hornero model eggs, while frequency of ejection of large cowbird model and control hornero model eggs did not differ. This result indicates that horneros are capable of discriminating cowbird model eggs from their own eggs when differences in size are large, but they fail to do so when differences are minor. This is what is expected when differences between host and parasitic eggs decrease, because the cost of miss-ejections as a result of recognition errors increases (Davies et al., 1996).

Our results are consistent with those of Mason and Rothstein (1986), who showed that horneros eject cowbird eggs using egg size as a cue and proposed that they use the width of the cowbird egg relative to the narrowest hornero egg of the clutch as the discrimination rule. In our study we expressed the length and width of the cowbird egg relative to the average length and width of the hornero clutch, as we consider this variable would be more appropriate if horneros have a template or mental image of their own eggs. Nevertheless, the use of either variable does not affect our qualitative results. In our study, the narrowest accepted experimental egg had a relative width (relative to the narrowest hornero egg of the clutch) of 0.89, which is remarkedly similar to the value of 0.88 reported by Mason and Rothstein (1986). Our results also indicate that the relative width would be a more accurate cue than the relative length, as the shortest accepted cowbird egg in our experiments had a relative length of 0.78.

Mason and Rothstein (1986) found that cowbird eggs in Uruguay, where the hornero is a common host of cowbirds, were 12 % larger than cowbird eggs in Buenos Aires, Argentina, where cowbirds rarely parasitize horneros. They proposed that in areas where horneros are frequent hosts of cowbirds, like Uruguay, the parasites evolved larger eggs to avoid the recognition and ejection of their eggs by horneros. They also showed that in Uruguay cowbird eggs laid in nests of horneros were longer than cowbird eggs laid in nests of other hosts but failed to find differences in egg width. We showed that the hornero was a common host in our study area and therefore could exert a selective pressure on cowbird egg size. We also showed that cowbird eggs laid in hornero nests were longer and wider than those laid in nests of chalk-browed mockingbirds, a sympatric host that does not eject cowbird eggs using egg size as a cue (Reboreda et al., 2013). A possible explanation for these differences would be the existence of host-specific female shiny cowbird lineages (Mahler et al., 2007; Domínguez et al., 2015). Females of the lineage that parasitizes horneros would have been selected for laying larger eggs, which decreases the probability of ejection by horneros. This selective pressure would be absent in females of the lineages that parasitize hosts that do not eject cowbirds eggs using size as a cue. Host-specific differentiation in egg size and egg shape in association with two divergent mitochondrial DNA lineages has also been reported in the brood parasitic greater honeyguide, *Indicator indicator* (Spottiswoode et al., 2011). Another possibility to explain the larger size of cowbird eggs in hornero nests would be that, because this host ejects small cowbird eggs, our data were biased (i.e. there were other small cowbird eggs laid in hornero nests that were ejected before we visited the nest). Although we cannot disregard this hypothesis, we consider it is less likely because our sample of cowbird eggs in hornero nests include eggs that were accepted and ejected (as well as others whose fate was unknown).

We also showed that horneros are capable of ejecting parasitic eggs even in the absence of their own eggs, which implies the use of a template-based recognition mechanism. This cognitive mechanism is frequent in hosts that reject parasite eggs based on differences in background eggshell colour or maculation (Victoria, 1972; Moskát et al., 2010; Bán et al., 2013; Moskát et al., 2014; Manna et al., 2017), but it has not been previously reported in hosts that use egg size as a discrimination cue. Because we do not know the age of the individuals that ejected the parasite eggs we cannot determine whether this template is innate or learned. However, the fact that males were also involved in egg ejection would indicate that the template is learned, as males should learn how the eggs of its partner are in the first reproductive attempt with its partner (Rodríguez-Gironés and Lotem, 1999; Stokke et al., 2007).

We observed that the frequency of ejection does not differ between sexes. Also, there were no sexual differences in other variables related to the ejection behaviour (Table 1). It is generally assumed that host females are responsible for egg ejection (Rothstein, 1975b; Davies and Brooke, 1988; Lotem et al., 1992; Palomino et al., 1998). This is because in many species females spend much more time in the nest than males. Sealy and Neudorf (1995) proposed that in species where males incubate or are more involved in attending the nest they would be more likely to evolve egg ejection. In agreement with this hypothesis, some studies showed that in species where females and males incubate, both sexes are ejectors (Soler et al., 2002; Lee et al., 2005) and Honza et al. (2007) reported that the probability ejection increases with the proportion of time one sex spends incubating. Our results are consistent with the hypothesis of probability of ejection increasing with the time each sex spends incubating, as in horneros both sexes contribute similarly to incubation (Massoni et al., 2012) and there were no sexual differences in probability of ejection.

In conclusion, our results provide new insights on the cognitive mechanisms underlying the recognition and ejection of parasitic eggs as the hornero is the first reported host that ejects parasite eggs using egg size as a cue paired with a template based cognitive mechanism. Besides, the frequency of ejection does not differ between sexes, which provides further evidence for equal egg rejection probability in species with egalitarian parental investment. Finally, the differences in egg size between cowbird eggs laid in hornero nests and those laid in nests of other hosts are consistent with the hypothesis of a host-specific female cowbird lineage evolving larger eggs as a counterdefense to deceit horneros from recognizing and ejecting parasitic eggs.

Author statement

RATG carried out field work, collected and analysed the data and wrote the first draft. BT developed the initial concept and hypothesis, help with funding acquisition, supervised field work and data analysis and wrote the first draft. JCR proposed the initial concept, developed the initial concept and hypothesis, supervised data analysis, wrote the initial and final drafts and made reviews and editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2020.104152.

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