



RESEARCH ARTICLE

Red-crested Cardinals use color and width as cues to reject Shiny Cowbird eggs

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ABSTRACT

As part of the coevolutionary process between brood parasites and their hosts, the latter have developed different strategies to discriminate and reject parasitic eggs. This recognition–rejection process is the primary host defense against costly brood parasitism. The Red-crested Cardinal (*Paroaria coronata*) is an occasional host of the generalist Shiny Cowbird (*Molothrus bonariensis*) that successfully rejects all parasitic eggs. We studied the cues used by Red-crested Cardinals to recognize and reject foreign eggs by experimentally adding real parasite and host eggs painted as mimetic or nonmimetic of host eggs and analyzing whether eggshell coloration and/or shape were used as cues for egg rejection. Rejection rates, mostly through egg ejection, were high for all nonmimetic eggs (95% for unpainted cowbird eggs and 100% for painted nonmimetic cowbird and host eggs). On the contrary, they were low for mimetic host eggs (6% for unpainted host eggs and 20% for painted mimetic host eggs), but intermediate for painted mimetic cowbird eggs (55%). We also found that egg width significantly affected the probability of rejection, with wider parasitic eggs (i.e. more different from host eggs) more frequently rejected. We report for the first time that egg width is an important cue for recognition and ejection of cowbird eggs in an open-cup-nesting host. Our results show that coloration is a reliable cue used by Red-crested Cardinals to discriminate and reject parasitic eggs, but when coloration alone does not allow discrimination of foreign eggs, this host uses egg width as an additional cue.

Keywords: antiparasitic defenses, brood parasitism, egg rejection, *Molothrus bonariensis*, *Paroaria coronata*, visual cues

Paroaria coronata usa como claves la coloración y el ancho para rechazar los huevos parásitos de *Molothrus bonariensis*

RESUMEN

Como parte del proceso coevolutivo entre los parásitos de cría y sus hospedadores, estos últimos han desarrollado diferentes estrategias para discriminar y rechazar los huevos parásitos. Este proceso de reconocimiento y rechazo es la principal defensa de los hospedadores frente al parasitismo de cría. *Paroaria coronata* es un hospedador ocasional de *Molothrus bonariensis* que rechaza exitosamente todos los huevos parásitos, pero las claves usadas para el reconocimiento de los huevos de *M. bonariensis* son aún desconocidas. En este trabajo estudiamos las claves usadas por *P. coronata* para reconocer y rechazar los huevos parásitos adicionando experimentalmente huevos naturales de la especie hospedadora y parásita pintados de forma mimética y no mimética y evaluando si la coloración y/o forma de los huevos eran usadas como claves para el rechazo. Las tasas de rechazo, mayormente por eyección del huevo, fueron altas para los huevos no miméticos (95% para los huevos parásitos sin pintar y 100% para los huevos parásitos y conespecíficos pintados no miméticos). Por el contrario, las tasas de rechazo fueron bajas para los huevos conespecíficos sin pintar (6%) y pintados de forma mimética (20%), pero intermedias para los huevos parásitos pintados miméticos (55%). También encontramos que el ancho de los huevos parásitos afectó significativamente la probabilidad de rechazo, siendo los más anchos (i.e. más diferentes a los huevos del hospedador) los más rechazados. Reportamos por primera vez que el ancho es una clave importante para el reconocimiento y rechazo de huevos parásitos en un hospedador de nido abierto. Nuestros resultados muestran que la coloración de los huevos es una clave confiable utilizada por *P. coronata* para discriminar y rechazar los huevos parásitos, pero cuando la coloración no permite la discriminación, usan el ancho de los huevos como una clave adicional.

Palabras clave: claves visuales, defensas antiparasitarias, *Molothrus bonariensis*, parasitismo de cría, *Paroaria coronata*, rechazo de huevos

INTRODUCTION

As part of the coevolutionary “arms race” between hosts and brood parasites (Dawkins and Krebs 1979, Rothstein 1990, Krüger 2007), hosts have developed various strategies to discriminate and reject parasitic eggs (Peer and Sealy 2004, Grim et al. 2011). Hosts frequently use 3 rejection strategies: nest abandonment, egg burial with nest material, and egg ejection (Davies 2000). The most studied strategy is the ejection of parasite eggs, whereby hosts discriminate and eject from the nest eggs dissimilar to their own eggs (Davies 2000, Underwood and Sealy 2006, Guigueno and Sealy 2012).

Numerous studies have shown that many hosts use egg coloration and spotting pattern to recognize foreign eggs (see Honza et al. 2007, Spottiswoode and Stevens 2010, Stoddard and Stevens 2011, Guigueno et al. 2014). In addition to egg coloration and spotting pattern, other attributes may help hosts recognize and reject parasite eggs, such as the size or shape of the latter in comparison to their own eggs (Marchetti 2000, Guigueno and Sealy 2012, Zölei et al. 2012, Guigueno et al. 2014). However, attributes such as egg size and shape have been considerably less studied than egg coloration and spotting pattern.

The Shiny Cowbird (*Molothrus bonariensis*) is a Neotropical, extreme generalist brood parasite whose eggs have been found in nests of >260 species (Lowther 2015). Shiny Cowbird eggs are highly variable in size, shape, background color, and spotting pattern, ranging from immaculate white to variable patterns of bright cinnamon brown spotting on a white background (Mahler et al. 2008, Gloag et al. 2014). Some hosts accept both immaculate and spotted eggs (Mason 1986, Massoni and Reboreda 1998), others accept spotted eggs only (Mason 1986, Mermoz and Reboreda 1994, Sackmann and Reboreda 2003, Astié and Reboreda 2005), and at least 2 hosts, the Rufous Hornero (*Furnarius rufus*) and the Red-crested Cardinal (*Paroaria coronata*), eject all Shiny Cowbird eggs (Mason and Rothstein 1986, Segura and Reboreda 2012). Of the Shiny Cowbird hosts in which egg ejection has been studied, the Rufous Hornero is the only species reported to use size as a cue, ejecting eggs that are smaller than its own (Mason and Rothstein 1986). It has been proposed that this could be an adaptation to the dark environment of the Rufous Hornero’s cavity nest interior, where egg coloration is not a reliable cue (Mason and Rothstein 1986). No open-cup-nesting host has yet been documented to use egg shape to discriminate and reject Shiny Cowbird eggs.

The Red-crested Cardinal (hereafter “cardinal”) is an open-cup nester and occasional host of the Shiny Cowbird (hereafter “cowbird”) (i.e. frequency of parasitism 7%; Segura and Reboreda 2012). From the human visual perspective, the coloration of cowbird eggs does not mimic that of cardinal eggs. The background color of cardinal

eggs is usually light grayish olive, light brownish olive, or light brownish cream, with heavily dark brownish or brownish olive spotting pattern covering the entire surface (Figure 1A). Also, cardinal eggs are normally narrower than cowbirds’ eggs (Segura and Reboreda 2012). This host does not exhibit aggressive behaviors toward female cowbirds, but quickly recognizes and rejects cowbird eggs of different color appearance (i.e. immaculate white, lightly spotted, and highly spotted; most of them typically nonmimetic of host egg appearance) added experimentally to the nests, but they do not reject natural conspecific eggs (Segura and Reboreda 2012). The latter indicates that the cardinal’s responses evolved in the context of interspecific (cowbird–cardinal) parasitism (see also Samas et al. 2014). Most rejections (98%) are by puncture ejection, but 2% of the rejections are by nest abandonment (Segura and Reboreda 2012). Although the cardinal appears to be an infrequent host, with an estimated 7% of nests parasitized, rapid egg rejection is likely to lead to underestimates of the true frequency of parasitism.

Previous experimental work (Segura and Reboreda 2012) was unable to elucidate the relative importance of color and shape as cues for recognition and rejection of parasite eggs. In the present study, we used artificial stimuli in parasitism experiments to establish the sensory thresholds of the cardinal’s discrimination abilities (see Hauber et al. 2015, Lahti 2015). We experimentally added natural cowbird and cardinal eggs artificially painted as both parasite (nonmimetic) and host (mimetic) eggs to elucidate the cues used by this host to recognize and reject foreign eggs. If cardinals use only egg color as a cue, we expected them to reject all nonmimetic colored eggs (natural and painted nonmimetic cowbird and painted nonmimetic cardinal eggs), whereas if they use only egg shape as a cue, we expected them to reject all cowbird but no cardinal eggs. Finally, if they use both color and shape as cues, we expected them to reject all nonmimetic colored eggs and painted color-mimetic cowbird eggs.

METHODS

Study Area

The study was conducted at “Estancia La Matilde” (35°20’S, 57°11’W) near the town of Punta Indio, Buenos Aires Province, Argentina. The study site is a flat area of ~400 ha within the Biosphere Reserve “Parque Costero del Sur” (MAB-UNESCO). It is a semi-open grassland with several low chains of woodlands mainly dominated by native tree species such as *Celtis ehrenbergiana*, *Scutia buxifolia*, and *Schinus longifolius*.

Study Species

The Red-crested Cardinal (Thraupidae; Burns et al. 2002) is a sexually monomorphic species that inhabits semi-open

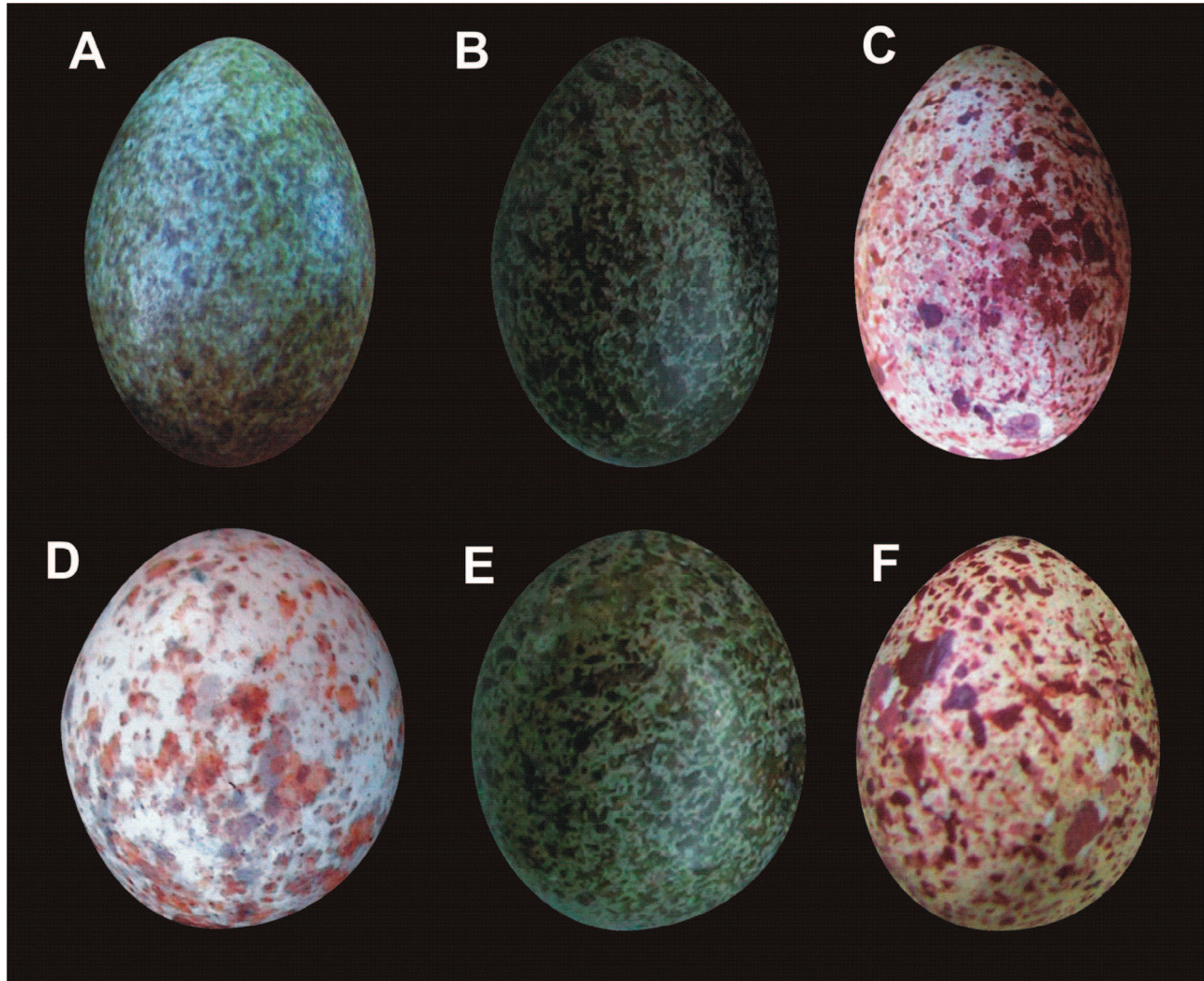


FIGURE 1. Experimental Red-crested Cardinal (host) and Shiny Cowbird (parasite) eggs added to nests of Red-crested Cardinals at Punta Indio, Buenos Aires Province, Argentina: (A) unpainted, (B) mimetic, and (C) nonmimetic-for-color host eggs; and (D) unpainted, (E) mimetic, and (F) nonmimetic-for-color parasite eggs.

areas with scattered trees and shrubs (Segura and Arturi 2012, Segura et al. 2014) from east central Argentina to southern Brazil, Paraguay, eastern Bolivia, and Uruguay (Ridgely and Tudor 2009). At our study site they breed from early October to late February and build open-cup nests. Modal clutch size is 3 eggs, and incubation starts with the laying of the second egg. Mean size of eggs is 25.2 ± 0.12 mm in length and 17.2 ± 0.08 mm in width (Segura et al. 2015).

Experimental Nests

We found nests by searching systematically in potential nest sites and by observing nesting behavior of territorial pairs. We monitored cardinal nests and carried out experiments of artificial parasitism in 40, 33, 36, and 24 nests during the breeding season in 2010, 2011, 2012, and

2013, respectively. Experimental nests were not manipulated in any way prior to experiments. As additional information, at 11 nests we video-recorded the first 4 hr after we added the experimental egg to study parents' behavior when encountering the foreign egg. The video camera was placed ≥ 3 m from the nest 2 hr before the experiment to habituate nest owners to its presence.

Experimental Eggs

Experimental eggs were painted with nontoxic acrylic paints (Eterna brand) to simulate the appearance of one of the cowbird morphs (heavily spotted with cream ground color; Figure 1D) and cardinal (Figure 1A) eggs. We used a mix of white (ID01), sienna (ID85), ocher (ID78), and olive (ID69) colors to achieve the ground-color and spotting-pattern effect on the egg surface. Cowbird and cardinal

experimental eggs were treated in 3 different ways (a total of 6 treatments): (1) unpainted, showing their natural color and spotting pattern: unpainted cardinal egg as positive control with similar shape and color (Figure 1A) and unpainted cowbird egg as negative control with different shape and color (Figure 1D); (2) painted to resemble cardinal eggs: color-mimetic cardinal egg with similar shape and color (Figure 1B) and color-mimetic cowbird egg with different shape and similar color (Figure 1E); and (3) painted to resemble cowbird spotted eggs: nonmimetically colored cardinal egg with similar shape and different color (Figure 1C) and nonmimetically colored cowbird egg with different shape and color (Figure 1F). We used natural cowbird eggs that were heavily spotted with cream ground color (Figure 1D). Also, experimental nonmimetically colored eggs were painted to resemble this morph (i.e. cream ground color with heavily brown spots; Figure 1C, 1F). All eggs were covered with matte water-based varnish (Eterna brand) after the pattern of each experimental treatment was applied, in order to protect the model surface and appearance from weathering. For each egg (both experimental and host eggs), we measured the width and length to the nearest 0.05 mm using Vernier calipers.

We collected fresh cardinal experimental eggs (i.e. without incubation) from nests that had been deserted during laying in our study site ($n = 18$ eggs) and from deserted nests ($n = 27$ eggs) from neighboring areas. Cardinal eggs used in experiments were 24.9 ± 0.14 mm (mean \pm SE; range: 22.6–27.3 mm) in length and 17.6 ± 0.06 mm (16.7–18.4 mm) in width, and there were no significant differences in length and width among experimental groups (Kruskal–Wallis tests; length: $H_2 = 2.67$, $P = 0.19$; width: $H_2 = 1.34$, $P = 0.51$; $n = 45$ eggs).

We collected fresh cowbird eggs (all of them heavily spotted with cream ground color; Figure 1D) from active Chalk-browed Mockingbird (*Mimus saturninus*; $n = 62$ eggs), Rufous-bellied Thrush (*Turdus rufiventris*; $n = 14$ eggs), and Rufous-collared Sparrow (*Zonotrichia capensis*; $n = 12$ eggs) nests present in the study area. Cowbird eggs used in the experiments were 23.5 ± 0.13 mm (20.4–26.0 mm) in length and 19.5 ± 0.15 mm (17.1–23.0 mm) in width, and there were no significant differences in length and width among eggs obtained from nests of different hosts (Kruskal–Wallis tests; length: $H_2 = 1.29$, $P = 0.52$; width: $H_2 = 2.62$, $P = 0.27$; $n = 88$ eggs) or among experimental groups (Kruskal–Wallis tests; length: $H_2 = 0.19$, $P = 0.99$; width: $H_2 = 1.77$, $P = 0.41$; $n = 88$ eggs).

Artificial Parasitism Experiments

We experimentally parasitized 133 nests with one cardinal egg ($n = 45$) or cowbird egg ($n = 88$). Of the cardinal eggs, 16 were unpainted, 20 were mimetic, and 9 were nonmimetic for color; whereas of the cowbird eggs, 21

were unpainted, 60 were mimetic, and 7 were nonmimetic for color. The different treatments were split evenly over the 4 yr, except for the treatment with cowbird nonmimetically colored eggs, which was split evenly over the first 3 yr. Nests were parasitized in the morning between 0600 and 0800 hours during the egg-laying ($n = 70$) and early incubation ($n = 63$) stages. In all cases, we approached the nest walking very slowly. In those cases in which the female was at the nest ($\sim 70\%$ of the visits), she left the nest when we were 10–12 m away. Because we did not record whether hosts were flushed prior to experimental parasitism, we could not control for this effect (see Hanley et al. 2015). Since cowbirds do not remove host eggs, we did not change host nest content at the time of experimental parasitism.

In all cases, we checked the nest for egg rejection at 1, 24, 48, and 120 hr after experimental parasitism. At each visit, we carefully examined host and parasite eggs for cracks or punctures and determined whether the nest was active or had been abandoned. We considered an experimental egg rejected if it disappeared from the nest (ejected) or if the nest was abandoned (i.e. eggs were cold and no parental activity was observed near the nest during 15–20 min). We considered an experimental egg accepted if it remained in the nest for ≥ 5 days after the experimental introduction (Rothstein 1974, Sealy 1996, Segura and Reboreda 2012).

Data Analysis

To determine whether painting the egg influenced rejection rates, we used a simple linear model to test rejection rates for (1) unpainted cardinal eggs (positive control) and mimetically painted cardinal eggs, and (2) unpainted cowbird eggs (negative control) and nonmimetically painted cowbird eggs.

We compared the outcomes across treatments using generalized linear models with logit link function and binomial error distribution. The response variable was the rejection of the egg (binary factor), and the fixed effects were color (mimetic or nonmimetic), egg type (cardinal or cowbird), year, nest stage (egg laying and early incubation), and clutch size (2, 3, and 4). We also included egg width and length (and the interactions with color and egg type), since there was no association between egg width and length variables (Spearman's rank correlation: $\rho = -0.03$, $P = 0.7$, $n = 133$). Additionally, we included an alternative predictor of shape calculated as the ratio width:length to test the effect of egg shape independently of the absolute values of width and length. We fitted generalized linear models using the maximum-likelihood approximation criterion. We used Akaike's Information Criterion (AIC) model selection, fitting all possible models using the package MuMIn in R 3.2.1 (R Development Core Team 2015). We report values as means \pm SE. Tests were 2-

TABLE 1. Numbers of nests at which experimental conspecific (Red-crested Cardinal) or parasite (Shiny Cowbird) eggs added to the nest were rejected either by ejection or nest abandonment at Punta Indio, Buenos Aires Province, Argentina. "Unpainted" eggs had the natural coloration, "mimetic" eggs were artificially painted as host eggs, and "nonmimetic" eggs were artificially painted as parasitic spotted eggs (see Figure 1).

	Ejected (n)	Abandoned (n)	Rejected (%)	Total (n)
Red-crested Cardinal eggs				
Unpainted	–	1	6	16
Mimetic	4	–	20	20
Nonmimetic	9	–	100	9
Shiny Cowbird eggs				
Unpainted	16	4	95	21
Mimetic	31	2	55	60
Nonmimetic	6	1	100	7

tailed, and differences were considered significant at $P < 0.05$.

RESULTS

Rejection rates of experimental cardinal and cowbird eggs for each treatment are detailed in Table 1. Most rejections (66 of 74 = 89%) were through egg ejection. At 38 of 66 nests (57%), ejection of experimental eggs occurred during the first hour, at 25 nests (38%) within 24 hr following experimental parasitism, and at 3 nests (5%) between 24 and 48 hr following experimental parasitism.

Painting did not affect the probability of rejection (unpainted vs. painted color-mimetic cardinal eggs: $Z = -0.004$, $P = 0.99$; unpainted vs. painted nonmimetic cowbird eggs: $Z = -1.12$, $P = 0.26$). Because of the lack of significant differences between painted and unpainted eggs, we pooled natural cardinal eggs with mimetically painted cardinal eggs, and natural cowbird eggs with nonmimetically painted cowbird eggs.

We found that the best-fitting model (based on AIC selection) included color, egg type, and the interaction between egg type and width (AIC_c value of the top model = 104.18; Δ AIC_c with second-best-fitting model [color, egg type, length, and the interaction between egg type and width] = 0.91; Δ AIC_c > 2 for subsequent models). Rejection of foreign eggs was significantly related to egg color and egg type (Table 2). Rejection rates were always high for nonmimetically colored eggs (>98%) and mostly low for color-mimetic eggs (<27%), although rejection of color-mimetic cowbird eggs was 55% (Table 1). Also, rejection rates were mostly high for cowbird eggs (>83%). Rejection rates were also significantly related to egg width (Table 2). In mimetically painted cowbird eggs, rejection rates varied with egg width but not with egg length (Figure 2); wider eggs (i.e. more different from host eggs) were

TABLE 2. Statistics and parameter estimates for predictors of rejection rates of experimental eggs added to 133 natural Red-crested Cardinal nests at Punta Indio, Buenos Aires Province, Argentina. "Color" included mimetic or nonmimetic eggs. "Egg type" included Red-crested Cardinal or Shiny Cowbird eggs. Generalized linear models were fit by the maximum-likelihood approximation criterion, with logit link function and binomial error distribution. "Rejection" (binary factor) was the response variable.

Rejections	B	SE	Z	P
Intercept	8.18	1.02	-1.81	0.082
Color	-4.51	1.18	-3.79	<0.001
Egg type	12.57	4.12	2.34	0.019
Width	4.45	2.11	1.84	0.065
Egg type * width	-5.68	2.41	-2.32	0.020

more frequently rejected. We found no effect of the egg shape predictor (width:length) or of year, nest stage, or clutch size on rejection rates.

Video recordings showed that when all adults returned to the nest they began pecking vigorously at the experimental egg. At 2 of the nests, instead of removing the experimental egg immediately, the bird pecked several times and then began incubating the eggs. This process happened several times throughout the recording period, but 24 hr later (when the video camera was not recording) the experimental egg was not in the nest. We did not observe any case of damage of host eggs in association with the ejection of the parasite egg, and we detected only one case (over the 133 experiments) of disappearance of one host egg in association with the acceptance of a mimetically painted conspecific egg.

DISCUSSION

Our results indicate that cardinals use both egg color and width as sensory cues to recognize and reject experimental eggs artificially added to natural nests, as they were able to eject most of the experimental nonmimetically colored eggs added to the nests and accepted similarly shaped mimetically colored eggs, thus supporting our third prediction.

Coloration seems to be a widespread cue used by hosts of the Brown-headed Cowbird (*M. ater*) to recognize and reject parasite eggs, as reviewed by Guigueno et al. (2014). Similarly, egg color is the cue used by hosts of the Shiny Cowbird that reject immaculate white eggs (i.e. Mason 1986, Mermoz and Reboreda 1994, Sackmann and Reboreda 2003, Astié and Reboreda 2005, de la Colina et al. 2012). Cardinals also use egg color to reject cowbird eggs. In the present study, they rejected all nonmimetically colored eggs (natural and nonmimetically painted cowbird and cardinal eggs). However, when egg coloration did not allow discrimination of foreign eggs (i.e. color-mimetically

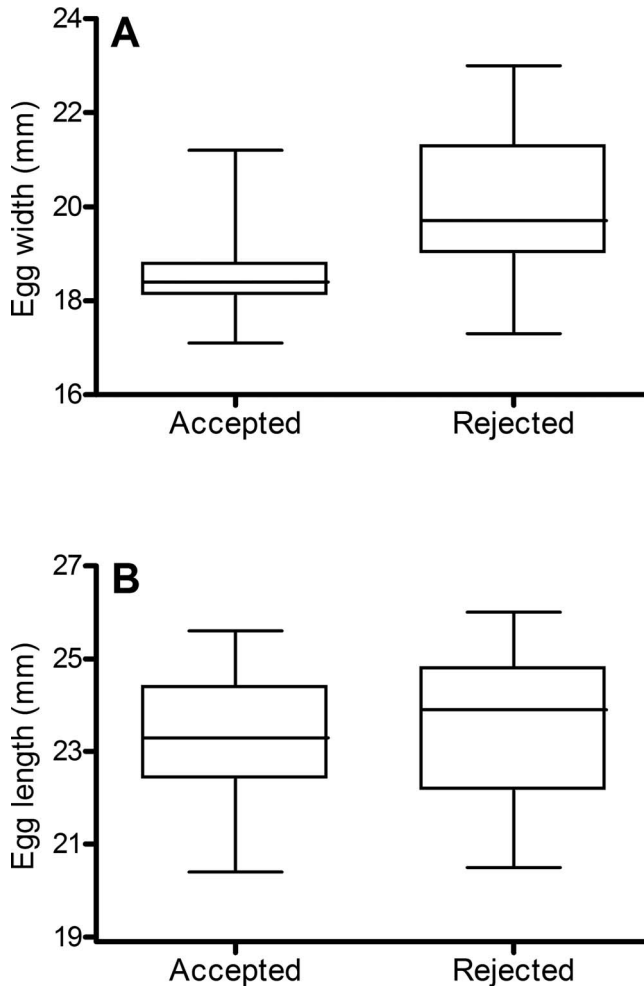


FIGURE 2. Box-and-whisker plots showing median, lower, and upper quartile, and smallest and largest (A) width and (B) length observations, for rejected ($n = 33$) and accepted ($n = 27$) mimetic-for-color Shiny Cowbird eggs in nests of Red-crested Cardinals at Punta Indio, Buenos Aires Province, Argentina.

painted cowbird eggs), cardinals used sensory cues from egg width as they were able to recognize and eject those eggs that were wider than their own eggs. Similarly, Polačiková and Grim (2010) and Zölei et al. (2012) reported that egg shape (estimated from asymmetric differences between the egg's blunt and sharp poles) played an important role in discrimination of own and foreign eggs by several host species parasitized by the Common Cuckoo (*Cuculus canorus*).

It has been reported that another Shiny Cowbird host, the Rufous Hornero, assesses the overall egg-size variation in its clutch and ejects cowbird eggs that have widths <88% of the width of its own eggs (Mason and Rothstein 1986). For this cavity-nesting host, egg color is a cue not available in the dark environment of the nest interior. Our results show, for the first time, that egg width is also an

important cue for recognition and ejection of cowbird eggs in an open-cup-nesting host.

As previously reported (Segura and Reboreda 2012), our results confirmed that cardinals are puncture ejectors and do not have important costs associated with the ejection of parasite eggs, such as the destruction of their own eggs. Although previous studies have found that the risk of mistakenly damaging the host's own egg is significantly greater with puncture ejection than with grasp ejection (Lorenzana and Sealy 2001), cardinals can remove parasite eggs without damaging their own eggs. We detected only one case among 133 experiments in which one host egg disappeared in association with the acceptance of a mimetically painted conspecific egg. In this case, we cannot rule out the possibility that this disappearance was the result of a puncture event by a female cowbird that visited but did not parasitize the nest (Gloag et al. 2013), followed by ejection of the damaged egg. Thus, our results confirm that in this puncture ejector, the cost of ejection of foreign eggs is negligible. We highlight the importance of using real eggs instead of artificial eggs in parasitism experiments (see also Briskie 2003, Prather et al. 2007), because the use of the latter may underestimate the frequency of rejection of parasite (Martín-Vivaldi et al. 2002) or conspecific eggs (Samas et al. 2014) and artificially increase the costs of egg ejection.

Evidence of immediate egg recognition was apparent at the video-recorded nests. In all these nests, when one of the birds returned to the nest, it pecked at the experimental egg repeatedly, indicating immediate recognition of the egg as foreign. Although in most cases (9 of 11 nests) the adult quickly removed the experimental egg, in 2 nests there was immediate recognition but the adult ejected the experimental egg >4 hr after experimental parasitism. This suggests that ejection behavior, at least in some individuals, may develop progressively beyond the initial recognition of foreign eggs (see also Lang et al. 2014).

We found that while nonmimetically colored eggs led to almost full rejection of experimental eggs, mimetic eggs did not necessarily lead to full acceptance since half of cowbird mimetically colored eggs were rejected on the basis of egg width. Thus, our results indicate that cardinals have a multi-cue decision-making mechanism to recognize and reject foreign eggs, both egg coloration and width having an important weight in the cognitive decision rules for rejection. A multi-cue decision-making process has also been shown in other cowbird hosts (de la Colina et al. 2012). The maintenance of the ability to recognize more than one sensory cue could be an adaptive advantage of Red-crested Cardinals that enables them to better avoid brood parasitism, given the large degree of polymorphism in Shiny Cowbird eggs.

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Author contributions: L.N.S., B.M., and J.C.R. conceived the study. L.N.S. designed the methods. L.N.S. and F.G.DiS. performed the experiments. L.N.S., F.G.DiS., B.M., and J.C.R. analyzed the data. L.N.S., F.G.DiS., B.M., and J.C.R. wrote the paper. B.M. and J.C.R. contributed substantial resources or funding.

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