

A test of the nest sanitation hypothesis for the evolution of foreign egg rejection in an avian brood parasite rejecter host species

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Abstract Hosts of avian brood parasites have evolved diverse defenses to avoid the costs associated with raising brood parasite nestlings. In egg ejection, the host recognizes and removes foreign eggs laid in its nest. Nest sanitation, a behavior similar in motor pattern to egg ejection, has been proposed repeatedly as a potential pre-adaptation to egg ejection. Here, we separately placed blue 3D-printed, brown-headed cowbird (*Molothrus ater*) eggs known to elicit interindividual variation in ejection responses and semi-natural leaves into American robins' (*Turdus migratorius*) nests to test proximate predictions that (1) rejecter hosts should sanitize debris from nests more frequently and consistently than accepter hosts and (2) hosts that sanitize their nests of debris prior to the presentation of a foreign egg will be more likely to eject the foreign egg. Egg ejection responses were highly repeatable within individuals yet variable between them, but were not influenced by prior exposure to debris, nor related to sanitation tendencies as a whole, because nearly all individuals sanitized their nests. Additionally, we collected published data for eight different host species to test for a potential positive correlation between

sanitation and egg ejection. We found no significant correlation between nest sanitation and egg ejection rates; however, our comparative analysis was limited to a sample size of 8, and we advise that more data from additional species are necessary to properly address interspecific tests of the pre-adaptation hypothesis. In lack of support for the nest sanitation hypothesis, our study suggests that, within individuals, foreign egg ejection is distinct from nest sanitation tendencies, and sanitation and foreign egg ejection may not correlate across species.

Keywords Nest sanitation · Egg rejection · Brood parasitism · *Turdus migratorius*

Introduction

Interspecific avian brood parasites avoid raising their own offspring by laying their eggs in the nests of host species (Davies 2000). Hosts may accept the eggs and incur costs of incubating and raising the foreign offspring, or hosts may avoid these costs by rejecting foreign eggs, removing the nestlings of brood parasites from their nest, or abandoning the nest and re-nesting elsewhere (Payne 1977). Egg rejection is the most common defense against brood parasitism (Rothstein 1975a; Medina and Langmore 2016). A host may reject foreign eggs by ejecting the eggs from the nest, burying eggs within the nest, or deserting the nest entirely (Rothstein 1975a). Egg ejection occurs when a host grasps or pierces an egg with its bill and carries it away (i.e., ejects) from the nest. Egg ejection is likely to be the most adaptive form of egg rejection, because it requires the least amount of time and energy in comparison to other rejection behaviors (Rothstein 1975a). However, ejection can be costly if egg recognition errors are made and a host rejects its own eggs (Davies et al.

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1996). Therefore, the ability to recognize foreign eggs and eject them from the nest is under strong selection in host species of brood parasites.

A similar behavioral motor pattern to egg ejection occurs when birds sanitize their nests of hatched egg shells, fecal sacs, and leaf and twig debris by grasping them with the bill and carrying them away from the nest. Nest sanitation is an adaptive behavior that may reduce risk of nest predation by ridding the nest of conspicuous cues (Blair and Tucker 1941; Tinbergen et al. 1962), reduce the degree of exposure to parasites and pathogens in the nest (Thomson 1934), and avoid loss of offspring to egg-capping (Hauber 2003). Nest sanitation behavior has been suggested to have been coopted (or a “pre-adaptation”; sensu Gould and Vrba 1982) for the evolution of egg ejection behavior by hosts of brood parasites (Rothstein 1975a; Moskát et al. 2003a; Guigueno and Sealy 2012; Poláček et al. 2013; Yang et al. 2015a). Additionally, although there are multiple classes of egg rejection behaviors (e.g., burial or nest desertion), egg ejection is the rejection behavior that most closely resembles nest sanitation, and therefore, it is the relevant form of egg rejection that may have evolved from nest sanitation (Rothstein 1975a).

The nest sanitation hypothesis posits that “rejecter and acceptor [species] should, respectively, show intense and weak nest sanitation behavior” (Rothstein 1975a, p. 265). In other words, if egg ejection has evolved from nest sanitation, then rejecter hosts should express nest sanitation behavior more frequently and consistently than acceptor hosts. The brown-headed cowbird (*Molothrus ater*; hereafter “cowbird”) is a generalist brood parasite with many different host species. Nearly all individuals of a particular host species either accept or reject cowbird eggs, and host species are, respectively, classified as “accepters” or “rejecters” (Rothstein 1975b). Yet, the evidence supporting a potential evolutionary and/or proximate link between nest sanitation and egg ejection within this brood parasite-host system is inconclusive. On the one hand, nest sanitation behavior may not be a significant interspecific correlate of egg rejection behavior across different host species of the brown-headed cowbird (Peer and Sealy 2004; Rothstein 1975a), but a potential correlation between continuous rates of egg rejection and sanitation has yet to be explicitly tested for. On the other hand, within species, nest sanitation behavior has been suggested as having some influence on egg rejection behavior in red-winged blackbirds (*Agelaius phoeniceus*), yellow-headed blackbirds (*Xanthocephalus xanthocephalus*), yellow warblers (*Setophaga petechia*), gray catbirds (*Dumetella carolinensis*), and American robins (*Turdus migratorius*) (Ortega and Cruz 1988; Guigueno and Sealy 2009; Underwood and Sealy 2006) (Table 1). There are also varied pieces of evidence suggesting support for the nest sanitation hypothesis in other host-parasite systems, including intraspecific parasitism in Eurasian tree sparrows (*Passer montanus*) and interspecific *Cuculus* cuckoo parasitism in two species of *Hirundo* swallows (Poláček et al. 2013; Yang et al. 2015a, b) (Table 1).

Here, we tested the covariation between sanitation and egg ejection behaviors in the American robin, a rejecter host species that consistently responds to natural cowbird parasitism through ejecting parasitic eggs from the nest (Rothstein 1975a; Rasmussen et al. 2009).

In areas of sympatry with breeding populations of brown-headed cowbirds, American robins eject nearly 100% of cowbird eggs that are deposited in their nests, and individuals’ responses to cowbird eggs are invariable (Briskie et al. 1992; Peer and Rothstein 2010). However, when American robins are experimentally parasitized with artificial cowbird eggs, their rejection rates depend on model egg color and are repeatable within individuals (Croston and Hauber 2014a, b). Model egg colors that elicit intermediate rejection rates (40–80%; Røskaft et al. 2002) can be used to reveal underlying variation in foreign egg rejection thresholds between individuals and consistency of egg rejection responses within individuals (Samaš et al. 2011). The probability that a particular individual will reject or accept a foreign egg may depend upon the individual’s ability to obtain, absorb, and use cues about changes in the nest (i.e., cognitive disposition or phenotype) (Bán et al. 2013; Dukas 2004; Mendelson et al. 2016). Additionally, an individual’s disposition toward detecting changes around the nest may also carry over into sanitizing their nest of foreign debris. Here, we parasitized that American robin nests experimentally using a 3D-printed model cowbird egg painted a color that was demonstrated by Croston and Hauber (2014a, b) to be rejected at an intermediate rate (58%) in order to generate considerable variation in robins’ foreign egg rejection responses and test how individuals’ egg rejection thresholds may relate to their sanitation tendencies.

In this study, we tested predictions of the nest sanitation hypothesis sensu Rothstein (1975a) that egg ejection positively covaries with and evolved from nest sanitation at both the intraspecific and interspecific levels. Mainly, do rejecter hosts that eject foreign eggs from their nest also sanitize their nests of debris more frequently and consistently than acceptor hosts? Secondly, are individual hosts more likely to reject foreign eggs following repeated exposure to debris in their nests? At the intraspecific level, if nest sanitation motivation (or tendency to remove debris from nests) is proximately linked with egg ejection behavior, then individual American robin females that frequently and consistently sanitize their nests of natural debris should also eject foreign eggs more often than females that do not sanitize their nests of debris. Additionally, if exposure to foreign debris in nests primes hosts to remove foreign eggs from the nest, then American robin females exposed to debris before being experimentally parasitized with a foreign egg should be more likely to eject the foreign egg than female robins that are exposed to debris following an experimental parasitism trial. If American robin (hereafter “robin”) females’ sanitation tendencies are not

Table 1 Comparison of nest sanitation and egg ejection studies by host species studied

Host species	Host category ^a	Brood parasite species	Test ‘parasite’ egg used	Foreign objects used	Control object/egg	Percent of test eggs ejected (n)	Percent of foreign objects removed (n)	Stated hypothesis	Predictions	Results support proximate predictions for host category? ^b	Results support pre-adaptation predictions for host category? ^c	Source
American robin	Rejecter	Brown-headed cowbird	White plaster pointed and rounded eggs	White wooden spheres, cylinders, rectangles, and cubes	White plaster robin-shaped egg	37.2 (43)	59.5 (42)	Object shape plays a role in egg discrimination	Responses to objects in nest vary by object shape	–	Yes	Underwood and Sealy (2006)
		Brown-headed cowbird	Blue brown-headed cowbird-sized, 3D-printed model egg	Blue <i>Douglas fir</i> leaf clippings	3D-printed mimetic robin egg	65.3 (75)	97.7 (77)	Sanitation motivation is a proximate influence of egg rejection behavior in individual hosts	Rejecter individuals sanitize nests more than accepters	No	Yes	This study
Barn swallow	Accepter, but authors re-identify as an intermediate rejecter citing data from Liang et al. (2013)	Common cuckoo	Non-mimetic blue clay model egg	Peanut shells	–	38.7 (62)	100 (25)	Nest sanitation elicits egg discrimination	Egg rejection will be higher in combination with nest sanitation than without it	Yes ^d	No	Yang et al. (2015b)
		Common cuckoo	Non-mimetic blue clay model egg	Blue clay coin and stick models, peanut shells	–	48.4 (62)	80.4 (138)	Nest sanitation is a pre-adaptation to egg rejection	Populations with higher egg rejection rates reject more non-egg-shaped objects	Yes ^e	Yes ^f	Yang et al. (2015a)
Great reed warbler	Intermediate rejecter	Common cuckoo	Polyester model cuckoo egg	Polyester sticks and coins	–	43.1 (51)	80.2 (96)	(1) Removal of non-egg-shaped objects is nest sanitation, (2) object removal is a specific anti-brood parasite defense	(1) Non-egg-shaped objects will be rejected more often than egg-shaped objects, (2) more parasitic eggs will be rejected than non-egg-shaped objects	–	Yes	Moskát et al. (2003a)
Gray catbird	Rejecter	Brown-headed cowbird	Blue-green plaster pointed and rounded eggs	Blue-green wooden spheres, cylinders,	Blue-green plaster catbird-shaped egg	24.1 (42)	58.1 (43)	Object shape plays a role in egg discrimination	Responses to objects in nest vary by object shape	–	No	Underwood and Sealy (2006)

Table 1 (continued)

Host species	Host category ^a	Brood parasite species	Test 'parasite' egg used	Foreign objects used	Control object/egg	Percent of test eggs ejected (n)	Percent of foreign objects removed (n)	Stated hypothesis	Predictions	Results support proximate predictions for host category? ^b	Results support pre-adaptation predictions for host category? ^c	Source
Red-rumped swallow	Acceptor	Common cuckoo	Non-mimetic blue clay model egg	rectangles, and cubes Blue clay coin and stick models	-	0 (14)	17.9 (28)	Nest sanitation is a pre-adaptation to egg rejection	Barn swallows reject foreign eggs at a higher rate than red-rumped swallows	-	Yes	Yang et al. (2015a)
Red-winged blackbird	Acceptor	Brown-headed cowbird	Model eggs of various sizes and colors, cowbird models, red-winged blackbird models	White and blue pom-poms, large and small beads, oblong models, stars	Real cowbird eggs, real red-winged blackbird eggs	23 (178)	98 (43)	Post hoc nest sanitation elicits egg ejection in an acceptor species	None stated	-	No	Ortega and Cruz (1988)
Yellow warbler	Acceptor	Brown-headed cowbird	Real brown-headed cowbird egg	Cowbird-color wooden stars and dumbbells	Real yellow warbler egg	3.5 (200)	5.9 (287)	Yellow warblers remove non-egg-shaped objects to clean nests	Before and during incubation, objects will be ejected more than objects resembling eggs	-	Yes	Guigneno and Sealy (2009)
Yellow-headed blackbird	Acceptor	Brown-headed cowbird	Model eggs of various sizes and colors, cowbird models, red-winged blackbird models	White and blue pom-poms, large and small beads, oblong models, stars	Real cowbird eggs, real yellow-headed blackbird eggs	8.4 (83)	100 (6)	<i>Post hoc</i> : Nest sanitation elicits egg ejection in an acceptor species	None stated	-	No	Ortega and Cruz (1988)

^a Host categories are based on rates of ejection of real and model brood parasite eggs: rejecter >80%, intermediate rejecter 40–80%, and acceptor <40% (see Rothstein 1975a, b and Roskoff et al. 2002)

^b Proximate predictions: foreign object removal and egg ejection should positively covary according to *individuals'* host categories. Rejecter individuals should sanitize their nests more frequently than acceptor individuals

^c Pre-adaptation (ultimate) predictions: foreign object and egg ejection should covary positively according to *species'* host categories. Rejecter species should sanitize their nests more frequently than acceptor species, and intermediate rejecter species should sanitize their nests at an intermediate rate (40–80%; Roskoff et al. 2002)

^d Comparing a group that had an opportunity to sanitize nests vs. a separate group that had no opportunity to sanitize nests

^e Comparing different barn swallow populations

^f In comparison between barn swallows and red-rumped swallows

related to their acceptance/ejection responses to foreign eggs in the nest, and exposure to debris in nests does not prime robins to eject foreign eggs, then we may rule out motivation to remove debris from the nest as a prominent proximate influence on foreign egg recognition and ejection in individual robins.

In contrast, at the species level, nest sanitation may be a pre-adaptation for egg ejection behavior if most individuals of this rejecter species sanitize their nests frequently. Additionally, in order to supplement this study and review previous studies testing the nest sanitation hypothesis, we also provide a comparative test of whether nest sanitation positively correlates with egg ejection among multiple host species of avian brood parasites (Table 1 and Fig. 4).

Materials and methods

Study area and subjects

The study took place at three separate tree farms near Champaign-Urbana, IL, USA, during the robin breeding season (May–June) in 2015. Cowbirds and robins are sympatric at all study sites (AB Luro, personal observation; Sauer et al. 2014). The main subjects of the study were nesting adult robin females that were laying and incubating. Active robin nests ($n = 35$) were monitored daily. Nests were located by searching every planted tree at each site systematically. After finding an active nest that contained one or more eggs, each egg was numbered with a permanent marker (black Sharpie™) and the nest was assigned a unique ID for differentiation. Nest ID was used as a proxy for individual female identity. By and large, only female robins, not males, incubate eggs in the nest (Howell 1942; Young 1955; Martin 1973), and only females reject eggs in host species where females alone incubate (Rothstein 1970, 1975a; Palomino et al. 1998; Amundsen et al. 2002; Soler et al. 2002; Samaš et al. 2011). We observed an absence of natural cowbird parasitism in our study population (no cowbird eggs found in $n = 89$ nests), but note that the absence of observed nests that contained cowbird eggs may have been due to the prompt egg ejection by robins if and when cowbird parasitism occurred.

Two experiments were performed on each nest in duplicate, which are two trials of artificial brood parasitism (Fig. 1b) and two trials of nest debris (Fig. 1a). Trials of an experiment were consecutively completed, experiments were done separately from one another, and the order in which experiments were performed at nests was counter-balanced into two groups (i.e., group 1 artificial parasitism trials were completed before the nest debris experiment began; group 2 nest debris trials were completed before the artificial parasitism experiment began). In the transition from one experiment to the next, the second experiment began on the same day the first experiment ended. In cases where nests were depredated during experimental trial time

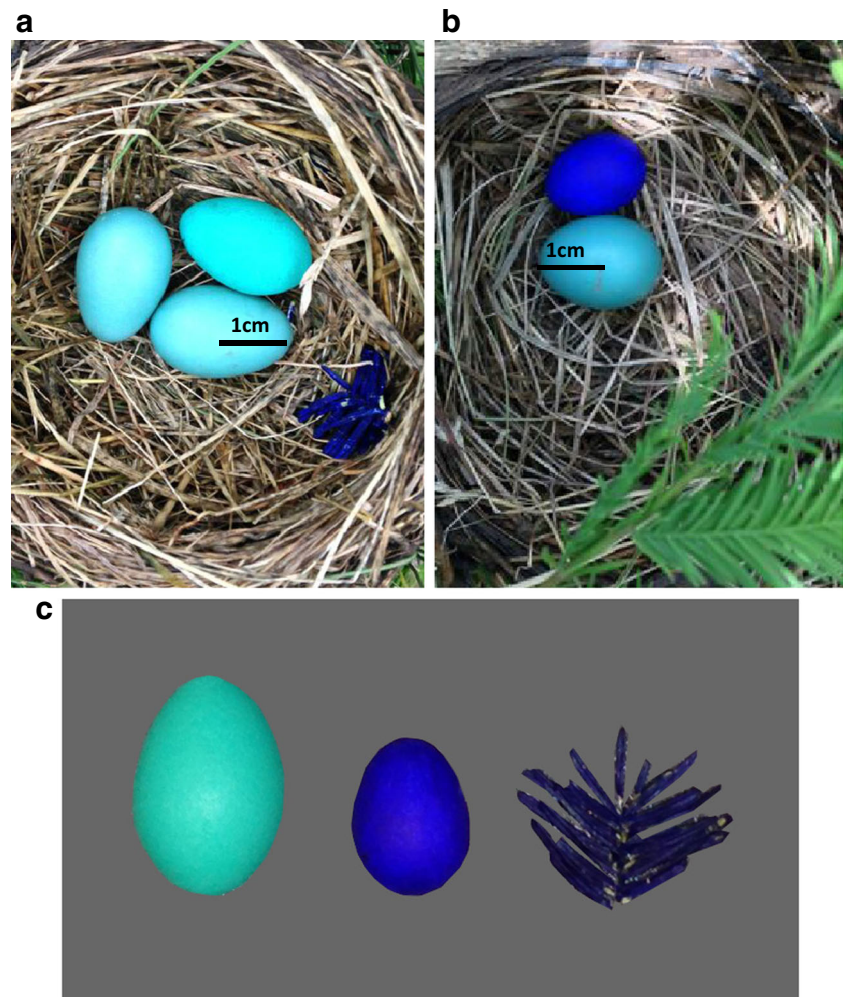
periods, the trial was ended and these data were not included in the analyses. Robins often re-nest close to the location of their previous failed nesting attempt (Howell 1942). Therefore, no new active nests located within 10 m of a depredated nest were tested, and exclusion of these nests greatly reduced the likelihood that the same unmarked individual birds were accidentally re-tested (Croston and Hauber 2014b). Conservatively, we estimated that it was possible for $n = 2$ nests to have been pseudoreplicated, assuming that the owner of a depredated nest re-nested at a distance >10 m from their original nest location at a given study site, and began laying eggs at a new nest after a minimum of 6 days since the date their first nest was depredated (see Electronic Supplementary File).

Two unincubated eggs were collected opportunistically (i.e., if nest was found during laying period) from most nests over the course of the study period. These eggs were collected for a separate study that investigated maternal investments in eggs. All eggs collected were replaced immediately with 3D-printed robin-size eggs painted a mimetic natural robin egg color (see “AMRO Ground” in Croston and Hauber 2014a), which were placed carefully in the same location and position as the collected eggs. None of the mimetic robin eggs were rejected during the study. Prior work in other *Turdus* thrushes found no significant effect on egg rejection responses when artificial eggs were replaced or added to nests (Moskát et al. 2003b; Honza et al. 2005, 2007). Nests where no eggs were collected were also analyzed (no eggs collected, $n = 7$; eggs collected, $n = 30$), and there was no significant effect of egg collection on acceptance/rejection of experimental cowbird eggs (no eggs collected = 72% rejection, eggs collected = 67% rejection; $X^2 = 0.59$, $df = 1$, $P = 0.99$, 3000 Monte Carlo replicates, likelihood ratio test) or nest sanitation responses (no eggs collected = 86% sanitation, eggs collected = 100% sanitation; $X^2 = 4.05$, $df = 1$, $P = 0.20$, 3000 Monte Carlo replicates, likelihood ratio test).

Tests for egg ejection and nest sanitation

All nests found were monitored daily; therefore, the nest stage (i.e., laying or incubating) was known within 1–3 days of nest monitoring for each nest. Our procedures for artificial parasitism followed those of Croston and Hauber (2014a) and Igc et al. (2015). At the start of an artificial parasitism trial, a 3D-printed, brown-headed cowbird-sized egg (<http://www.shapeways.com/product/9B4PARXSD/cow-bird-egg-smooth>) that was painted a blue color known to elicit an intermediate rejection rate of 58–70% (non-toxic Winsor and Newton Galeria Ultramarine Blue © acrylic paint) was inserted into the nest. The dimensions of the 3D-printed cowbird eggs are all within the range of the natural variation of real cowbird eggs (Ankney and Johnson 1985), 22.5 mm in length, 16.9 mm in breadth, and 3.1 g in mass (Fig. 1b; for measurements, see Igc et al. (2015), and for painting details, see

Fig. 1 Photos of experiments and objects. **a** Nest sanitation trial. **b** Artificial parasitism trial. **c** Experimental objects from *left to right* 3D model mimetic robin egg, 3D model *blue* cowbird egg, and *blue*-painted douglas fir leaf clipping



Croston and Hauber (2014a)). Each nest was checked daily to mark any additional eggs, check the presence of previously marked eggs, and observe if the artificial cowbird egg was rejected or remained in the nest. Robins are known to reject model cowbird eggs via ejection, so the absence of the blue model cowbird egg from the nest was recorded as an ejection (Rothstein 1975a, b, 1982; Rasmussen et al. 2009). The 3D-printed model eggs used could not be punctured, so all recorded ejections were deemed as grasp ejections (documented by Igc et al. 2015). Robins may have attempted to puncture-eject the 3D cowbird model eggs, but we could not determine if they attempted to do so. Trials where eggs hatched or the nest was depredated (i.e., all eggs absent, often some egg remains present within and/or nearby nest) were not included in the analyses. Over 80% of cowbird model eggs are rejected within 2 days (Aidala et al. 2015), so the presence of the model egg in the nest after 3 days was recorded as an acceptance (Croston and Hauber 2014a). The second trial began the day the first model egg was rejected or after the first model egg remained in the nest for 3 days. If the model egg remained in the nest after 3 days, it was deemed accepted, removed by the

experimenter from the nest, and another model egg was added to begin the second trial.

Douglas fir (*Pseudotsuga menziesii*) leaf clippings of near-uniform size (measured to 31.44-mm² rectangle; comparable to surface area of artificial cowbird eggs, 22.5 × 16.9 mm), shape, and color were inserted into the nest on the inside nest lining (Fig. 1a). Leaf clippings were painted the same blue color as the blue model cowbird eggs used in the artificial parasitism experiment to control for any properties of the blue paint that might influence rejection/sanitation responses (Fig. 1c). The leaf's presence was noted during daily nest monitoring. Nest sanitation was recorded nominally as absence of the leaf from the nest. If the leaf remained in the nest after 3 days, the response was recorded as no sanitation. The second trial began the day the first leaf was no longer present or on the third day if the first leaf remained. If the first leaf was still present in the nest on the third day, it was removed by the experimenter, and another painted leaf was inserted into the nest to begin the second nest debris trial.

As a control to test robins' responses to the addition of an egg to the nest, a single 3D-printed mimetic robin egg

(Fig. 1c) was added to a separate group of nests ($n = 5$). At another separate group of nests, eggs were moved slightly and a piece of nest material was lifted gently and replaced to test robins' responses to nest visits/manipulations per se ($n = 5$).

Statistical analyses

We included 35 separate nests in all analyses (artificial parasitism, $n = 35$; nest clutter $n = 32$). Of the 35 nests, 30 completed all experimental trials and 5 completed both trials of one experiment and at least one trial of the other experiment (e.g., completed artificial parasitism trials 1 and 2, nest sanitation trial 1) before hatching or nest depredation interrupted experimentation. Our analyses included an assessment of individual repeatability of egg ejection and sanitation behavior, use of generalized linear mixed models (GLMMs) to test if individuals' responses were specific toward the type and order of experiment performed while also examining potential effects of confounding covariates, and a test of correlation between nest sanitation and egg ejection behavior across $n = 8$ different avian brood parasite host species.

Repeatability may be defined as the portion of variation in a trait which may be attributed to variation among individuals, and is a suitable proxy of the heritability of a behavior (Wolak et al. 2012). Following Samaš et al. (2011) and Croston and Hauber (2014b), we performed two statistical tests to assess repeatability of foreign egg ejection and nest sanitation responses and check if individual robins could be categorized as consistent accepters vs. rejecters and sanitizers vs. non-sanitizers, which are a Spearman's correlation between binary egg ejection responses and a single-score intraclass correlation coefficient (ICC) for a one-way model using the irr package in R v3.2.4 (R Core Team 2016; Gamer et al. 2012).

We also used a GLMM with a Laplace approximation using the lme4 package in R v3.2.4 (Bates et al. 2015) to test if robins' egg ejection and sanitation responses could be differentiated by the type of experiment performed (artificial parasitism vs. nest clutter) and/or were affected by the order in which the experiments took place (i.e., test for a priming effect of one experiment taking place before the other) while accounting for potential confounding factors. We ran multiple generalized linear mixed models and detected the most parsimonious GLMM using the Akaike information criterion for small sample sizes (AIC_c) (Burnham and Anderson 2002). Our GLMM on responses to experiments included nest ID as a random effect; fixed effects of experiment type (artificial parasitism vs. nest clutter) and order of experiment; and additional fixed effects of potential confounding factors including clutch size, nest stage (laying vs. incubation), and whether or not the experimenter flushed robins from the nest to begin the experimental trial (Hanley et al. 2015). Additionally, we ran a separate set of GLMMs looking only at responses to artificial parasitism trials in order to specifically test the effects of

confounding factors on egg ejection responses alone. Date was also included in our initial models, but it did not improve any of the models—therefore, we did not include it in our final model set.

Lastly, to test our interspecific predictions, we performed two Spearman's rank correlation tests comparing rates of removal of foreign objects from nests with egg ejection rates using data from previous studies ($n = 8$ different species; Table 1 and Fig. 4), one comparing raw rates and one comparing rates with species-independent contrast transformations. Because closely related species with common ancestors cannot be considered independent data points within statistical analyses (Felsenstein 1985), a correction can be made by calculating the differences (contrasts) between phenotypic trait values of closely related species using an available phylogeny (i.e., subtracting trait values from tip to tip down a phylogeny) (Felsenstein 1985; Garland et al. 1992). Ejection rates with species-independent contrasts were calculated as outlined in Garland et al. (1992).

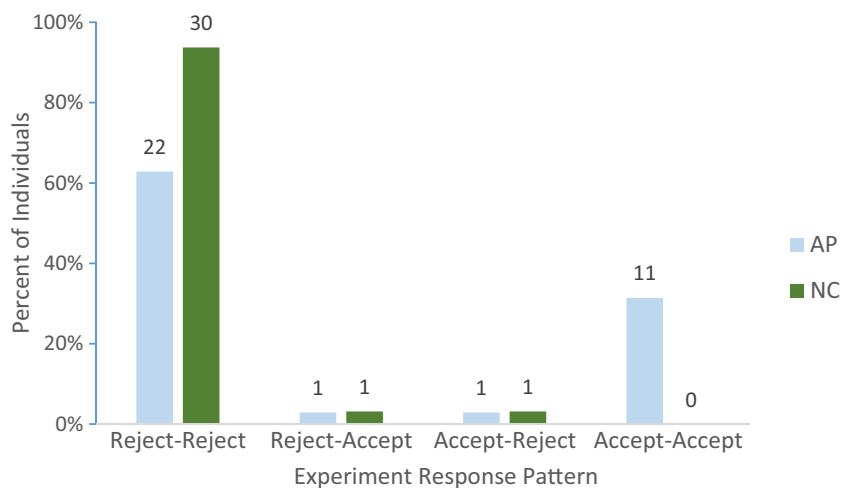
All statistical tests were done in R v3.2.4 (R Core Team 2016). For the phylogeny used for calculation of independent contrasts, we obtained 100 trees from birdtree.org (Jetz et al. 2012) and then acquired a consensus tree using Mesquite v.3.04 (Maddison and Maddison 2016).

Results

Across all artificial parasitism and nest clutter trials, we detected the following patterns: 65% of blue cowbird model eggs were ejected and 97% of blue Douglas fir leaves were removed from nests (Fig. 2). Thirty three of 35 females were consistent in their responses to artificial parasitism (11 as repeated accepters and 22 as repeated rejecters; Fig. 2), suggesting high interindividual repeatability (Spearman's correlation $r_s = 0.87$, 95% CI = 0.76–0.93, $n = 35$, $P < 0.0001$; ICC = 0.87, 95% CI = 0.77–0.94, $F = 15.2$, $n = 35$, $P < 0.0001$; Fig. 3). Thirty of 32 females were consistent in their responses to nest clutter trials (all 30 as repeated rejecters). The repeatability of sanitation behavior was not significant because there was almost no variation in sanitation responses among individuals (Spearman's correlation $r_s = -0.03$, 95% CI = -0.38–0.32, $n = 32$, $P = 0.86$; ICC = -0.016, 95% CI = -0.36–0.33, $F = 0.97$, $n = 32$, $P = 0.536$; Fig. 3).

The best GLMM predicting combined responses to both artificial parasitism and nest sanitation experiments using AIC_c values included only nest ID and experiment type as predictors ($w_i = 0.29$; Table 2). Three other models also had $\Delta_i < 2$, but 95% CIs of model-averaged parameter estimates overlapped 0 for all fixed effects except experiment type (i.e., whether the experimenter flushed the bird from the nest, order of experiment, and clutch size), so models containing these fixed effects did not have useful predictive value (Burnham and Anderson 2002). For GLMMs predicting responses to

Fig. 2 Responses of individuals to artificial parasitism (AP) and nest clutter (NC) experiments ($n = 2$ trials per experiment). Number of individual hosts exhibiting response pattern is noted above bars



artificial parasitism trials alone, only nest ID was included in the best model ($w_i = 0.42$; Table 2). Among artificial parasitism response models, there was a second model with $\Delta_i < 2$, but the 95% CI model-averaged parameter estimate for the fixed effect of flushing in this model included 0, so its overall effect was considered negligible.

Finally, there was no significant correlation between rates of removal of foreign objects vs. ejection of model eggs from nests among host species of obligate brood parasites (unaltered species-level Spearman’s correlation, $r_s = 0.38$, 95% CI = $-0.33-0.81$, $n = 10$, $P = 0.27$; independent contrast Spearman’s correlation, $r_s = 0.38$, 95% CI = $-0.38-0.83$, $n = 9$, $P = 0.31$; Fig. 4).

Discussion

We inserted blue artificial cowbird eggs and leaf debris into American robins’ nests as separate experiments with duplicate trials, and we did not find support for our individual-level

proximate link predictions of Rothstein’s (1975a) nest sanitation hypothesis. Specifically, individual robins’ egg ejection responses were distinct from their nest sanitation responses (Table 2). The ejection/acceptance of artificial cowbird eggs was highly repeatable (i.e., consistent within individual robins, yet variable between them), but nest sanitation was invariable across nearly all contexts and subjects (Figs. 2 and 3). These results demonstrate that (1) between individuals, foreign egg recognition and ejection thresholds are variable while nest sanitation is consistent, and (2) within individuals, both egg ejection and sanitation decision thresholds are relatively fixed. These findings suggest that nest sanitation is a highly conserved behavior of American robins that may currently be under stronger selection than and unrelated to foreign egg recognition and ejection in this rejecter host species. However, these conclusions may be idiosyncratic to the blue-painted leaf stimulus used, because most robins removed it from their nest. Future studies may benefit from using an intermediately removed semi-natural sanitation stimulus, along with an intermediately rejected model egg, and testing

Fig. 3 Repeatability of nest sanitation and egg rejection behaviors as 95% confidence intervals of the intraclass correlation coefficients (ICCs) of responses to repeated trials. Sample sizes of individual robins tested are noted above intervals

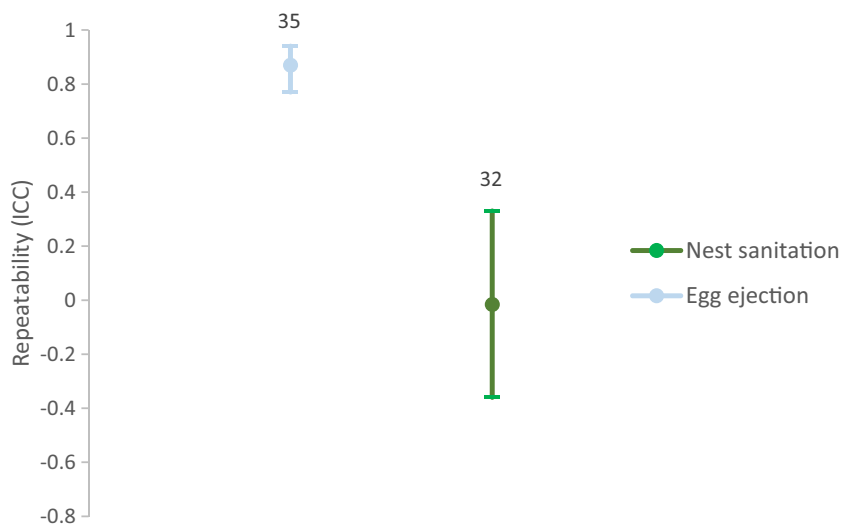


Table 2 GLMMs of American robins' responses to (1) artificial parasitism and nest sanitation experiments and (2) only the artificial parasitism experiment

Models	<i>K</i>	<i>AIC_c</i>	Δ_i	<i>w_i</i>
Artificial parasitism and nest sanitation responses (<i>n</i> = 35)				
Nest ID + experiment type	3	89.14	0	0.29
Nest ID + flushed + experiment type	4	89.36	0.22	0.26
Nest ID + order of experiment + flushed + experiment type	5	90.37	1.23	0.15
Nest ID + clutch + nest stage + flushed + experiment type	6	90.66	1.52	0.13
Nest ID + clutch + flushed + experiment type	5	91.16	2.02	0.10
Nest ID + nest stage + order of experiment + flushed + experiment type	6	92.07	2.93	0.07
Nest ID + clutch × nest stage × order of experiment + flushed + experiment type	11	100.69	11.55	0.00
Nest ID	2	138.2	49.06	0.00
Artificial parasitism responses alone (<i>n</i> = 35)				
Nest ID	2	53.79	0	0.42
Nest ID + flushed	3	54.30	0.51	0.33
Nest ID + clutch + flushed	4	56.21	2.42	0.13
Nest ID + clutch + order of experiment + flushed	5	58.02	4.23	0.05
Nest ID + nest stage + clutch + flushed	5	58.22	4.43	0.05
Nest ID + nest stage + order of experiment + flushed	5	58.81	5.02	0.03
Nest ID + nest stage × clutch × order of experiment × flushed	10	74.51	20.72	0.00

Nest ID nest identity as a random effect; fixed effects include *experiment type* artificial parasitism experiment or nest sanitation experiment, *flushed* whether or not the experimenter flushed the robin away from its nest to begin an experimental trial, *order of experiment* ordinal position in which experimental trial took place, *clutch* clutch size at start of experimental trial, *nest stage* whether robin was in laying or incubation stage at start of trial. Models are ordered by their estimated quality according to Akaike information criteria corrected for small sample sizes (*AIC_c*) (Burnham and Anderson 2002) from top to bottom (highest to lowest quality). *K* number of model parameters, Δ_i *AIC_c(i) - AIC_c(min)*, *w_i* Akaike weights

individuals repeatedly with both stimuli in order to assess potential covariation between sanitation response and egg ejection thresholds across individuals.

We found that eliciting nest sanitation behavior does not prime hosts to be more likely to eject foreign eggs; the best

model predicting egg rejection responses did not include the order in which artificial parasitism and nest sanitation experiments took place (artificial parasitism responses only; Table 2). Our findings are in contrast with Yang et al.'s (2015b) recent study of barn swallows, but unlike their study

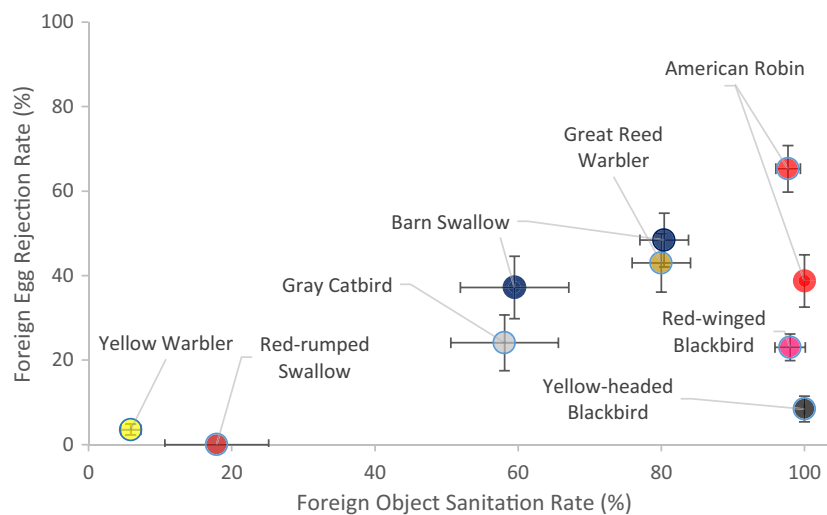


Fig. 4 Scatterplot of foreign egg ejection vs. foreign object sanitation rates for *n* = 8 different avian brood parasite host species (*n* = 5 *Molothrus ater* hosts, *n* = 3 *Cuculus canorus* hosts). Rates were obtained from previous studies as well as the current study (see Table 1 for the rates).

Error bars are ± binomial SE as percentages. Raw species-level Spearman's correlation $r_s = 0.38$, 95% CI = -0.33–0.81, *n* = 10, *P* = 0.27. Independent contrasts Spearman's correlation $r_s = 0.38$, 95% CI = -0.38–0.83, *n* = 9, *P* = 0.31

which presented sanitation and egg rejection stimuli simultaneously, our methodology allowed for the explicit examination of the influence of timing and order of egg ejection vs. nest sanitation; we performed artificial parasitism and nest clutter experiments separately in two counterbalanced-order treatment groups instead of simultaneously presenting model eggs with debris in nests. We conclude that foreign egg recognition and ejection are distinct from nest sanitation motivation and that they remain relatively consistent and repeatable within individual hosts regardless of whether or not the hosts have recently sanitized their nests of debris.

Nest sanitation is a ubiquitous behavior in American robins, a rejecter host species of the brown-headed cowbird, and this finding is consistent with pre-adaptation predictions of Rothstein's (1975a) nest sanitation hypothesis at the species level. However, we found no significant correlation between foreign egg ejection and nest sanitation rates in our phylogenetic comparative analysis of eight host species (Fig. 4). Host species that consistently eject foreign eggs may exhibit highly frequent and consistent nest sanitation behavior as a consequence of having evolved an enhanced cognitive ability, selected for under the pressure of brood parasitism, which has a positive feedback effect on both egg ejection and nest sanitation (Bán et al. 2013) (e.g., heightened sensitivity for detecting any visual or tactile changes around the nest). It is also possible that nest sanitation and egg ejection are unrelated and that foreign egg recognition and ejection evolved independently of nest sanitation. Furthermore, the majority of nest sanitation behavior probably involves removal of egg shells and feces after eggs have hatched naturally (Tinbergen et al. 1962; Wallace and Mahan 1975; Blair and Tucker 1941; Guigueno and Sealy 2012). Because most interspecific brood parasites deposit their eggs during the laying and incubation stages of hosts, ejections of most foreign eggs occur before eggs begin to hatch. Nest sanitation was not a significant correlate of egg ejection behavior in our cross-species analysis of obligate brood-parasite hosts, yet previous studies insist that nest sanitation may be a pre-adaptation for anti-brood parasite defenses (Fig. 4 and Table 1). However, our interspecific comparisons are confined to only eight different host species from seven separate studies, where different types of debris and foreign egg stimuli were inserted into nests. In response to these inconsistencies, we advocate that robust phylogenetic comparisons of multiple species across brood parasite-host systems are needed before any definitive conclusions can be made about the role and directionality of nest sanitation in the evolution of foreign egg ejection. Additionally, we suggest that future studies replicate our methods in other brood parasite host species to improve the quality and power of phylogenetically informed, comparative tests across different host species.

Akin to our study, future experimental work investigating the nest sanitation hypothesis should use ecologically

relevant, semi-natural stimuli to delineate clearly nest sanitation from egg ejection and consider the costs and benefits of each behavior within the context of the studied host species' ecologies. In a review of the relevant experimental literature (Table 1), we found that past sanitation hypothesis studies focused on differences and similarities of hosts' responses to *various experimental objects* placed in nests rather than measuring separately the variation in egg ejection and nest sanitation behavior *within the hosts themselves* (see also Guigueno and Sealy 2012), did not utilize ecologically relevant stimuli for measuring natural nest sanitation (i.e., removal of eggshells, fecal sacs, and debris), and did not have proper control objects (i.e., model conspecific eggs; see Table 1). The insertion of various artificial objects differing in their degrees of egg-like appearance into nests to examine how sanitation relates to egg ejection poses two problems: (1) the researcher defines when an observed behavior is egg ejection or sanitation based on how egg-like the objects appear from the human perspective and (2) a sanitation hypothesis which assumes that egg ejection and sanitation share a common discrimination threshold based on external stimulus' features cannot be falsified based on behavioral data alone (i.e., neural activation pattern comparisons are necessary). In the past studies examined, sanitation stimuli did not differ properly from model eggs used in the number of sensory parameters (size, shape, color, material, texture, etc.) to properly investigate a cognitive task of nest sanitation separate from foreign egg recognition. In addition, the degree to which the model eggs' color mimics either the host's or its brood parasite's eggs should also be carefully considered. Here, we have made an *umwelt* gamble in our use of artificial blue model cowbird eggs and blue-painted leaf clippings, but have carefully considered their use based on past work (Croston and Hauber 2014a, b) and believe it to be justified for the purposes for this study (Hauber et al. 2015; but see Lahti 2015). Finally, we recommend that future tests of the nest sanitation hypothesis measure the potential costs and benefits of nest sanitation directly and compare them with those of egg ejection to assess the adaptive relevance of each behavior to the host species of interest (e.g., compare sanitized vs. unsanitized nests, ectoparasite loads in nests, number of offspring fledged, and predation rates).

In American robins, an individual's motivation to sanitize foreign debris from its nest seems to have little influence on the likelihood of recognizing a foreign egg in the nest and ejecting it. In accordance with the sanitation pre-adaptation hypothesis predictions, robins are a rejecter host species of brown-headed cowbirds that exhibit highly frequent and consistent sanitation behavior. However, the prediction that rejecter host species should exhibit greater sanitation than acceptor host species across eight different host species was not statistically supported when controlling for phylogenetic relatedness (Fig. 4). In conclusion, we did not find evidence in

support of the nest sanitation hypothesis at the proximate or ultimate level, more data and replication of egg ejection and sanitation experiments across many host species are needed, and robust analyses using phylogenetic comparative methods are necessary before any definitive conclusions can be made about potential evolutionary patterns between nest sanitation and foreign egg ejection.

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Compliance with ethical standards

Ethical standards All experiments and procedures of this study were IUACUC approved (MH 2/16-T3) and complied with US laws.

Conflict of interest The authors declare they have no conflict of interest.

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