Investigating the nest sanitation hypothesis in a rejecter species: is sanitation a proximate link, pre-adaptation, or by-product of egg ejection?

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Investigating the nest sanitation hypothesis in a rejecter species: is sanitation a proximate link, pre-adaptation, or by-product of egg ejection?

by

Alec B. Luro

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of the requirements for the degree of
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Abstract

Hosts of 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 experimentally tested the prediction that rejecter hosts should sanitize their nests of debris more frequently and consistently than accepter hosts at the individual level by placing blue 3D-printed brown-headed cowbird (*Molothrus ater*) eggs and semi-natural leaves into American robins’ (*Turdus migratorius*) nests. Egg ejection and sanitation responses were highly consistent within individual robins, but the two behaviors did not positively covary. At the individual level, our results do not support the prediction that sanitation tendencies have a proximate influence on egg ejection decisions. At the species level, our experimental findings do support pre-adaptation hypothesis predictions for this rejecter host. However, when we combined our data with previous sanitation hypothesis studies to compare sanitation and egg ejection rates across eight host species, we found no support for the pre-adaptation hypothesis predictions. Our study suggests that, proximately, foreign egg ejection is distinct from nest sanitation motivation within individuals, and ultimately, sanitation and foreign egg ejection do not correlate according to whether a host species is an accepter or rejecter. Therefore, we must also consider that heightened sanitation in rejecter host species may be a by-product of rather than a pre-adaptation for the evolution of egg ejection.
**Introduction**

Interspecific avian brood parasites forgo 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 2015). 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. 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 as an exaptation (or 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).

The nest sanitation hypothesis posits that “rejecter and accepter [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 nest sanitation should be a more expressed component in rejecter hosts’ behavioral repertoires than accepter hosts’. In hosts of the brown-headed cowbird (Molothrus ater) brood parasite system, 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, some of the applicability of the nest sanitation hypothesis in this system remains inconclusive. On the one hand, nest sanitation behavior is not a significant correlate of egg rejection behavior across different host species of the brown-headed cowbird (hereafter: cowbird) (Peer and Sealy 2004; Rothstein 1975a). On the other hand, 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 petechial), gray catbirds (Dumetella carolinensis) and American robins (Turdus migratorius) (Ortega and Cruz 1988; Guigueno and Sealy 2009; Underwood and Sealy 2006). 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, 2015b) (Table 1). Though there are multiple kinds of egg rejection behaviors, egg ejection is the only rejection behavior that resembles nest sanitation and, therefore, it is the relevant form of egg rejection that may have evolved from nest sanitation (Rothstein 1975a). Of the species listed
here, only gray catbirds and American robins are rejecter species that respond to natural parasitism with cowbird eggs via ejection consistently (Rothstein 1975a; Rasmussen et al. 2009). Therefore, American robins are suitable candidates for studies of egg ejection and nest sanitation, and this species is the main subject of our study.

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, 2014b). 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) (Bán et al. 2013; Dukas 2004). 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 American robin nests experimentally using a 3D-printed model cowbird egg painted a color that was demonstrated by Croston and Hauber (2014a, 2014b) 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 individual level proximate link and species level pre-adaptation predictions of the nest sanitation hypothesis \textit{sensu} Rothstein (1975a) that egg ejection evolved from nest sanitation. Mainly, do rejecter hosts that eject foreign eggs from their nest also sanitize their nests of debris more frequently and consistently than accepter hosts? If nest sanitation motivation is proximately linked with egg ejection behavior, then individual American robin females that sanitize their nests of natural debris should also eject foreign eggs more often than females that do not sanitize their nests of debris. Alternatively, we predict that nest sanitation motivation is not proximately linked with egg ejection behavior (i.e., the two behaviors do not covary). Although the motor components of these two behaviors are physically similar, their cognitive requirements and potential costs/benefits may be different. Egg ejection is a more complex cognitive task than nest sanitation behavior, because it requires the host to be able to discriminate between different types of eggs to avoid ejecting their own eggs (Bán et al. 2013). In addition, sanitation is an appropriate response to any object within the nest that is not egg-shaped (Moskát et al. 2003a; Underwood and Sealy 2006).

If American robin (hereafter: robin) females’ sanitation tendencies are not related to (covary with) their acceptance/ejection responses to foreign eggs in the nest, then we may rule out the cognitive mechanisms underlying nest sanitation motivation 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, and if nest sanitation correlates with egg ejection among multiple brood parasite host species (Table 1).
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. 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: 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 cases where nests were depredated during experimental trial time periods, the trial was ended and the data
were not included in the analyses. Robins often re-nest close to the location of their previous failed nesting attempt. Therefore, no new active nests located within 10m of a depredated nest were tested, and exclusion of these nests ensured that the same unmarked individual birds were not accidentally retested (Rothstein 1975b).

Two unincubated eggs were collected opportunistically from each nest 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 (N=7), and there was no significant effect of egg collection on acceptance/rejection of experimental cowbird eggs ($X^2=2.78$, $df=1$, $P=0.096$, likelihood ratio test) or nest sanitation responses ($X^2=0.33$, $df=1$, $P=0.57$, likelihood ratio test).

Tests for egg ejection and nest sanitation

Our procedures for artificial parasitism followed those of Croston and Hauber (2014a) and Igic et al. (2015). At the start of an artificial parasitism trial, a 3D-printed brown-headed cowbird-sized egg that was painted a blue color known to elicit an intermediate rejection rate of 58-70% (non-toxic Winsor & Newton Galeria Ultramarine Blue © acrylic paint) was inserted into the nest (for measurements, see Igic et al. 2015; for painting details, see 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, 1975b, 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 Igic et al. 2015). 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 leaf clippings (Pseudotsuga menziesi) of near-uniform size (measured to 31.44mm² rectangle; comparable to surface area of artificial cowbird eggs), shape, and color were inserted into the nest on the inside nest lining (Fig. 1B). 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. 1). 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 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. We performed likelihood ratio and Fisher’s exact probability tests to measure contingencies of rejection/acceptance responses to artificial parasitism trials and sanitation responses to nest clutter trials. We also ran likelihood ratio tests on responses to experiments and potential confounding factors, including: clutch size (a proxy of nest age), date of experiment, the order of the experiment, and whether or not the experimenter flushed robins from the nest to begin the experimental trial (Hanley et al. 2015).

Lastly, we performed two Spearman-rank correlation analyses that compared rates of removal of foreign objects from nests with egg ejection rates using data from previous studies (N=8 different species: Table 1): one comparing raw rates and one comparing rates with species-independent contrast transformations. Ejection rates with species-independent contrasts were calculated as outlined in Garland et al (1992).
All statistical tests were done using JMP v.11 (SAS 2013), and graphs were made using GraphPad Prism v.6.07 for Windows (GraphPad 2015). A continuous trait phylogenetic comparative tree was made by first obtaining 100 trees from birdtree.org (Jetz et al. 2012), obtaining a consensus tree using Mesquite v.3.04 (Maddison & Maddison 2001), and then using the `contMap` function from the package `phytools` in R v.3.2.4 (Revell 2013).

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), and 30 of 32 females were consistent in their responses to nest clutter trials (all 30 as repeated rejecters) (Fig.3). Likelihood ratio tests of responses to experiments yielded no significant effect of clutch size ($X^2=1.78$, $df=4$, $P=0.78$), date of experiment ($X^2=27.47$, $df=23$, $P=0.24$), order of the experiment ($X^2=0.76$, $df=4$, $P=0.94$), or whether or not the experimenter flushed robins from the nest ($X^2=0.33$, $df=1$, $P=0.57$). At control nests, no eggs were rejected and no nests were deserted (egg insertion, $N=5$; nest manipulation, $N=5$).

The only significant predictor of robins’ repeated egg ejection responses to artificial parasitism was their response in the prior artificial parasitism trial ($X^2=29.89$, $df=1$, $P<0.001$, Fisher’s exact test). In contrast, almost no variation was found in responses to nest clutter (Fig.2), thus, there were no significant predictors of sanitation responses to nest clutter trials (Table 2).
Finally, neither unaltered species level nor independent-contrast based comparisons between rates of removal of foreign objects vs. model eggs among hosts of obligate brood parasites significantly correlated (unaltered species level: Spearman correlation, $r_s=0.38, N=10$, $P=0.27$; independent contrasts: $r_s=0.38, N=9$, $P=0.31$) (see Table 1 for unaltered sanitation and egg ejection rates used; see Fig. 4 for visual representation of data).

**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 did not positively covary with their nest sanitation responses (Table 2). The ejection/acceptance of artificial cowbird eggs was consistent within individual robins, yet variable between them, but nest sanitation was invariable across nearly all contexts and subjects (Figs. 2, 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 and may currently be under stronger selection than foreign egg recognition and ejection in this rejecter host species. Nest sanitation and egg ejection behavior appear similar in motor functions, but are cognitively dissimilar; likely because they have different fitness costs and cognitive requirements, with egg ejection being the more risky and complex task of the two (Bán et al. 2013). Egg recognition is required for a host to selectively eject foreign eggs from the nest without committing recognition errors and ejecting its own eggs (Davies et al. 1996). Nest
Sanitation entails the removal of objects from the nest that are dissimilar to eggs (e.g., feces, debris, and egg shells); nest sanitation does not demand the same level of fine-tuned discrimination as egg recognition and ejection. Therefore, egg recognition and ejection do not positively covary with nest sanitation, and we validated this prediction experimentally.

We found that eliciting nest sanitation behavior does not prime hosts to be more likely to eject foreign eggs: among the subjects that received the nest sanitation trials first, we did not find greater egg rejection rates compared to subjects that received the experimental parasitism trials first (see Table 2, Response AP - Order of experiment: $P=0.99$). Our findings are in contrast with Yang et al.’s (2015b) recent study of barn swallows, but unlike their study 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 versus 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 species of cowbird eggs, and this finding is consistent with pre-adaptation predictions of Rothstein’s (1975a) nest sanitation hypothesis at the species level. Alternatively, heightened nest sanitation may not be a pre-adaptation, but rather a by-product egg ejection in rejecter species (Rothstein 1975a). We found no significant correlation between foreign egg ejection and nest sanitation rates in our phylogenetic comparative analysis of eight host species (Table 1 and Fig.4). Host species that consistently reject 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 1963; Wallace and Mahan 1975; Blair and Tucker 1941; Guigeno 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. We did not find nest sanitation to be 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). 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.

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 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. 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, 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; 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, and 2) a sanitation hypothesis which assumes egg ejection and sanitation share a common threshold based on external stimuli’s features cannot be falsified based on behavioral data alone. 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. True nest sanitation under natural circumstances involves the removal of natural debris, such as fecal sacs, ecto-parasites, detritus, egg shells, and unhatched eggs (Guigeno and Sealy 2012). The two behaviors and their cognitive requirements can only be delineated properly by using natural (or semi-natural) stimuli known to elicit nest sanitation behavior in natural scenarios (Lahti 2015; but see Hauber et al. 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 compare adaptive relevance of each behavior to the host species of interest (e.g., compare sanitized vs. un-sanitized nests: ecto-parasite loads in nests, number of offspring fledged, predation rates, etc.).

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 accepter host species did not hold true across eight different hosts species 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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Ethical standards

All experiments and procedures of this study were IUACUC-approved (MH 2/16-T3) and complied with U.S. laws. The authors declare they have no conflict of interests.
References


Figures and Tables

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, blue douglas fir leaf clipping.
Fig. 2 Rejection and acceptance responses to artificial parasitism (AP) and nest clutter (NC) experiments in the separate trials. Frequency of response is noted in parentheses.

Fig. 3 Combined responses to artificial parasitism and nest clutter experiments. Frequency of individual hosts exhibiting response pattern is noted in parentheses.
Fig. 4. Comparative phylogenetic tree of a. nest sanitation and b. foreign egg ejection rates for eight host species of obligate interspecific brood parasites. Z-scores of rates were used in figure and color scale is based on standardized rates. Foreign egg ejection and object sanitation rates used for figure were obtained from the following studies: Yellow Warbler (Guigeno & Sealy 2009), Yellow-headed Blackbird and Red-winged Blackbird (Ortega & Cruz 1988), American Robin and Gray Catbird (Underwood & Sealy 2006), Barn Swallow and Red-Rumped Swallow (Yang et al. 2015a), Great Reed Warbler (Moskát et al. 2003a).
Table 1 Comparison of nest sanitation and egg ejection studies by host species studied

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Host Category</th>
<th>Brood Parasite Species</th>
<th>Test &quot;Parasite&quot; Egg Used</th>
<th>Foreign Objects Used</th>
<th>Control Object/Egg</th>
<th>% Test Eggs Ejected (n)</th>
<th>% Foreign Objects Ejected (n)</th>
<th>Stated Hypothesis</th>
<th>Predictions</th>
<th>Results support proximate predictions for host category?</th>
<th>Results support pre-adaptation predictions for host category?</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Robin</td>
<td>Rejecter</td>
<td>Brown-Headed Cowbird</td>
<td>white plaster pointed and rounded eggs</td>
<td>white wooden spheres, cylinders, rectangles, and cubes</td>
<td>white plaster robin-shaped egg</td>
<td>37.2 (43)</td>
<td>59.5 (42)</td>
<td>Object shape plays a role in egg discrimination</td>
<td>Responses to objects in nest vary by object shape</td>
<td>---</td>
<td>yes</td>
<td>Underwood &amp; Sealy (2006)</td>
</tr>
<tr>
<td>Brown-Headed Cowbird</td>
<td></td>
<td></td>
<td>blue brown-headed cowbird sized 3D-printed model egg</td>
<td>blue douglas-fir leaf clippings</td>
<td>3D-printed mimetic robin egg</td>
<td>65.3 (75)</td>
<td>97.7 (77)</td>
<td>Sanitation motivation is a proximate influence of egg rejection behavior in individual hosts</td>
<td>Rejecter individuals sanitize nests more than accepters</td>
<td>no</td>
<td>yes</td>
<td>This study</td>
</tr>
<tr>
<td>Barn Swallow</td>
<td>Accepter, but authors re-identify as an Intermediate Rejecter citing data from Liang et al. (2013)</td>
<td>Common Cuckoo</td>
<td>non-mimetic blue clay model egg</td>
<td>peanut shells</td>
<td>____</td>
<td>38.7 (62)</td>
<td>100 (25)</td>
<td>Egg rejection elicits egg discrimination</td>
<td>yes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>no</td>
<td></td>
<td>Yang et al. (2015b)</td>
</tr>
<tr>
<td>Common Cuckoo</td>
<td></td>
<td></td>
<td>non-mimetic blue clay model egg</td>
<td>blue clay coin and stick models; peanut shells</td>
<td>____</td>
<td>48.4 (62)</td>
<td>80.4 (138)</td>
<td>Nest sanitation is a preadaptation to egg rejection</td>
<td>Populations with higher egg rejection rates reject more non-egg shaped objects</td>
<td>yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>yes&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Yang et al. (2015a)</td>
</tr>
<tr>
<td>Barn Swallow</td>
<td></td>
<td></td>
<td>Common Cuckoo</td>
<td>polyester model cuckoo egg</td>
<td>polyester sticks and coins</td>
<td>43.1 (51)</td>
<td>80.2 (96)</td>
<td>(1) Removal of non-egg shaped objects is nest sanitation (2) Object removal is a specific anti-brood parasite defense</td>
<td>(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</td>
<td>---</td>
<td>yes</td>
<td>Moskát et al. (2003a)</td>
</tr>
<tr>
<td>Species</td>
<td>Host Category</td>
<td>Parasite Model</td>
<td>Object Shape</td>
<td>Nest Sanitation</td>
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<tr>
<td>Gray Catbird</td>
<td>Rejecter</td>
<td>brown-headed cowbird</td>
<td>blue-green plastic pointed and rounded eggs</td>
<td>blue-green plastic catbird-shaped egg</td>
<td>24.1 (42)</td>
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<td></td>
<td>Object shape plays a role in egg discrimination</td>
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<tr>
<td>Red-rumped Swallow</td>
<td>Acceptor</td>
<td>common cuckoo</td>
<td>blue clay coin and stick models</td>
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<td>0 (14)</td>
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<td>Nest sanitation is a preadaptation to egg rejection</td>
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<tr>
<td>Red-winged Blackbird</td>
<td>Acceptor</td>
<td>brown-headed cowbird</td>
<td>white and blue pom-poms; large and small beads; oblong models; stars</td>
<td>real cowbird eggs; real red-winged blackbird eggs</td>
<td>23 (178)</td>
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<td>Post hoc: nest sanitation elicits egg ejection in an accepter species</td>
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<tr>
<td>Yellow Warbler</td>
<td>Acceptor</td>
<td>brown-headed cowbird</td>
<td>cowbird-color wooden stars and dumbells</td>
<td>real yellow warbler egg</td>
<td>3.5 (200)</td>
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<td>Yellow Warblers remove non-egg shaped objects to clean nests</td>
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<tr>
<td>Yellow-Headed Blackbird</td>
<td>Acceptor</td>
<td>brown-headed cowbird</td>
<td>white and blue pom-poms; large and small beads; oblong models; stars</td>
<td>real cowbird eggs; real yellow-headed blackbird eggs</td>
<td>8.4 (83)</td>
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<td>Post hoc: nest sanitation elicits egg ejection in an accepter species</td>
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</tbody>
</table>

1. Host categories are based on rates of ejection of real and model brood parasite eggs: rejecter >80%, intermediate rejecter 40-80%, accepter <40% (see Rothstein 1975a,b and Røskaft et al. 2002)
2. Proximate predictions: Foreign object and egg ejection should co-vary positively according to individuals' host categories. Rejecter individuals should sanitize their nests more frequently than accepter individuals
3. Pre-adaptation (ultimate) predictions: Foreign object and egg ejection should co-vary positively according to species' host categories. Rejecter species should sanitize their nests more frequently than accepter species, and intermediate rejecter species should sanitize their nests at an intermediate rate (40-80%; Røskaft et al. 2002)

a= comparing a group that had an opportunity to sanitize nests versus a separate group that had no opportunity to sanitize nests
b= comparing different barn swallow populations
c= in comparison between barn swallows and red-rumped swallows

Underwood & Sealy (2006)
Yang et al. (2015a)
Ortega & Cruz (1988)
Guigeno & Sealy (2009)
Ortega & Cruz (1988)
### Table 2: Results of contingency analysis for responses to artificial parasitism and nest sanitation experiments

<table>
<thead>
<tr>
<th>Comparison</th>
<th>df</th>
<th>$X^2$</th>
<th>Prob$&gt;X^2$</th>
<th>P-Value Fisher’s Exact Test</th>
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</thead>
<tbody>
<tr>
<td>Response AP 1-AP 2</td>
<td>1</td>
<td>29.89</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Response NC 1- NC 2</td>
<td>1</td>
<td>0.07</td>
<td>0.80</td>
<td>1</td>
</tr>
<tr>
<td>Response NC 2-AP 1</td>
<td>1</td>
<td>2.40</td>
<td>0.37</td>
<td>0.31</td>
</tr>
<tr>
<td>Response AP 2-NC 1</td>
<td>1</td>
<td>0.80</td>
<td>0.37</td>
<td>1</td>
</tr>
<tr>
<td>Response AP – Order of Experiment (1,2,3,4)</td>
<td>3</td>
<td>1.13</td>
<td>0.89</td>
<td>0.99</td>
</tr>
<tr>
<td>Outcome All Responses AP- NC</td>
<td>1</td>
<td>3.42</td>
<td>0.7552</td>
<td>0.58</td>
</tr>
</tbody>
</table>

AP= artificial parasitism, NC= nest clutter; 1, 2= trial number