OXFORD

Article

The ghosts of parasitism past: lingering frontline anti-brood parasite defenses in a former host

Matthew. H. J. Chaumont[a],*, Naomi E. Langmore[b], and Justin A. Welbergen[a]

[a]Hawkesbury Institute for the Environment, Western Sydney University Richmond, NSW, 2753, Australia, and

[b]Research School of Biology, Australian National University, Canberra, ACT 2600, Australia

*Address correspondence to Matthew H. J. Chaumont. E-mail: m.chaumont@westernsydney.edu.au

Abstract

Coevolutionary arms races between brood parasites and hosts provide tractable systems for understanding antagonistic coevolution in nature; however, little is known about the fate of frontline antiparasite defenses when the host "wins" the coevolutionary arms race. By recreating bygone species interactions, using artificial parasitism experiments, lingering defensive behaviors that evolved in the context of parasitism can be understood and may even be used to identify the unknown agent of parasitism past. Here we present the first study of this type by evaluating lingering "frontline" nest defenses that have evolved to prevent egg laying in a former brood parasite host. The Australian reed warbler *Acrocephalus australis* is currently not parasitized but is known to exhibit fine-tuned egg discrimination—a defensive behavior indicative of a past brood parasite–host arms race and common in closely related parasitized species. Here, using 3D-printed models of adult brood parasites, we examined whether the Australian reed warbler also exhibits frontline defenses to adult brood parasites, and whether we could use these defenses to identify the warbler's "ghost of parasitism past." Our findings provide evidence that the Australian reed warbler readily engages in frontline defenses that are considered adaptive specifically in the context of brood parasitism. However, individuals were unable to discriminate between adults of different brood parasite species at their nest. Overall, our results demonstrate that despite a relaxation in selection, defenses against brood parasitism can be maintained across multiple stages of the host's nesting cycle, and further suggest that, in accordance with previous findings, that learning may be important for fine-tuning frontline defense.

Key words: brood parasites, coevolutionary arms-race, ghosts of parasitism past, hosts, lingering traits

The interactions between avian brood parasites and their hosts have provided researchers with useful models for understanding coevolutionary processes in nature (Davies and Brooke 1989; Rothstein 1990). By laying eggs in a host's nest, brood parasites subject the host to the costs of raising unrelated offspring. In response, hosts evolve adaptations to deflect parasitism, which in turn select for further counter-adaptations in the parasite, and so on (Brooke and Davies 1988; Davies 2011; Soler 2014; Grim and Stokke 2016). Recent work evaluating the relationship between inter-specific obligate brood parasites and their hosts has shown that hosts use a "defense in depth strategy" against brood parasites (Welbergen and Davies 2009), whereby hosts deploy multiple lines of defense at successive stages of the nesting cycle, including at the "frontline," "egg," and "nestling" stages. Each defensive stage may be countered by brood parasite offenses,

providing the ingredients for coevolutionary arms races throughout the host nesting cycle (Britton et al. 2007; Welbergen and Davies 2009). In addition, it is now apparent that throughout the host nesting cycle, brood parasites use mimicry as a general strategy for circumventing discrimination mechanisms of their victims, whereas hosts counteract exploitation and improve the integrity of their defenses by fine-tuning their enemy recognition (Welbergen and Davies 2011). Although research has historically focused on the evolutionary outcomes from extant parasite–host interactions (reviewed in Feeney et al. 2014), and lingering egg stage defenses (Soler 2014), little else is known about the fate of antiparasite defenses when the host "wins" the arms race, as in when the brood parasite switches to another victim or goes extinct (Krüger et al. 2009; Soler 2014). This is an important issue because, in theory, lingering anti-brood parasite defenses may render former hosts impervious to future parasitism offenses (Rothstein 2001; Peer et al. 2011; Soler 2014) (below) and thus confound our understanding of the distribution of realized parasitism among potential hosts.

When a host wins the arms race against its brood parasite and the parasite stops attempting to parasitize the host, selection for its anti-parasite defenses becomes relaxed, so that the defenses may disappear from the system, particularly if they are costly (Lahti et al. 2009), risking re-exploitation (Soler 2014). Nevertheless, empirical studies on egg stage adaptations suggest that defenses that were once useful in the context of parasitism can persist well beyond the end of the coevolutionary arms race (Peer et al. 2011; Soler 2014), as long as these, what we call here "lingering defenses," are minimally costly (Rothstein 2001). Egg discrimination, the recognition, and rejection of eggs perceived as foreign, is exhibited as a specific response to brood parasitism (Davies and Brooke 1988); therefore, egg recognition and ejection costs are less likely to be realized in the absence of parasitism cues and so egg discrimination may persist after the arms race with a brood parasite has been won (Lahti et al. 2009). Though, egg rejection is likely to be more costly, being lost in the absence of parasitism at a faster rate (Soler et al. 2017; Yang et al. 2020). Alternatively, multiple species have been shown to retain antiparasite defenses despite the costs associated with egg discrimination (Samas et al. 2014). Accordingly, there is now ample evidence for finetuned egg discrimination in species currently free from brood parasitism (Welbergen et al. 2001; Lyon 2003; Lovászi and Moskát 2004; Martín-Vivaldi et al. 2013), for example, Soler (2014) showed that egg rejection has been retained in 54 (29.7%) of potential hosts. Across species, egg rejection rates of past (noncurrent, but otherwise suitable) hosts are similar to those of current hosts of evicting parasites and higher than those of current hosts of nonevicting brood parasites and of unsuitable hosts (Medina and Langmore 2015b). This suggests that lingering egg stage defenses are common behavioral anachronisms in former brood parasite hosts.

In contrast to lingering egg stage adaptations, evidence for lingering frontline adaptations is mixed. This is not surprising, because almost all research at the frontline (Feeney et al. 2012) has focused on extant arms races. However, red-backed shrikes *Lanius collurio* mob taxidermic cuckoo mounts of the common cuckoo *Cuculus canorus* at a significantly higher rate than they do controls in a population now abandoned by their former brood parasite (Lovászi and Moskát 2004). On the other hand, several European host species that were introduced to New Zealand without their brood parasite (common cuckoo, *C. canorus*) ~130 years ago, currently show little aggression toward taxidermic cuckoo mounts at their nests, in contrast to their counterparts in Europe (Hale and Briskie 2007 but see Grim and Stokke 2016). These, and other cases suggest that in some systems nest defenses can be expressed independently of prior individual experience (Veen et al. 2000; Roskaft et al. 2002; Lovászi and Moskát 2004; Saunders et al. 2013), whereas in others, they must be acquired or enhanced through learning (Lindholm and Thomas 2000; Welbergen and Davies 2012; Langmore et al. 2012; Feeney and Langmore 2013; Kuehn et al. 2016). Further studies indicate that both experience-independent and experience-dependent mechanisms can contribute simultaneously to the expression of frontline defenses (Wiebe 2004; Welbergen and Davies 2012). Regardless, frontline defenses can in theory be retained irrespective of their mode of acquisition, although for experience-dependent mechanisms it would be the learning ability rather than the defensive behavior *per se* that would be expected to linger. To our knowledge, no studies have attempted to teach former hosts to mob their former brood parasites. Therefore, although there is experimental evidence for lingering frontline defenses in some cases, in others, evidence of absence may be confounded by a necessity for learning.

Lingering anti-brood parasite defenses are an important study subject as they can help explain why various otherwise suitable hosts are currently unparasitized (Soler et al. 1999; Berg et al. 2006; Feeney et al. 2014). Furthermore, if the lingering defenses involve a functionally referential component, such as frontline discrimination against specific brood parasites or egg stage discrimination against specific egg morphs, then these can help expose, through behavioral experimentation, the identity of the brood parasite(s) responsible for these "ghosts of parasitism past." Thus, by studying anti-brood parasitism defenses that have evolved as a response to coevolutionary arms races between brood parasites and former hosts we can potentially gain important information about how long hosts remain resistant to parasitism, and identify key features of the hosts past selective environment, such as the identity and tactics of the past parasite, how the host comes to identify its antagonist's, and the role of learning in nest defense.

Previous work (e.g., Welbergen et al. 2001) demonstrated that the Australian reed warbler *Acrocephalus australis* exhibits finely tuned egg discrimination, suggestive of an evolved response to parasitism as observed in other species (Rothstein 1990; Langmore et al. 2005; Lahti 2006; Medina and Langmore 2015a; Medina and Langmore 2015b). Furthermore, there is anecdotal evidence that the reed warbler also exhibits frontline and chick stage defenses (Welbergen J.A. et al. unpublished data), all traits suggestive of a protracted brood parasite–host coevolutionary interaction. However, unlike many of its congeners, the Australian reed warbler is not currently hosted to a specific brood parasite, despite the facts that (1) it is sympatric with 7 species of brood parasitic cuckoo (Brooker and Brooker 1989; Welbergen et al. 2001), (2) its breeding ecology is similar to other parasitized reed warbler species (Schulze-Hagen and Leisler 2011), and (3) there is no evidence of conspecific parasitism (Brooker and Brooker 1989; Berg 1998). Therefore, the Australian reed warbler provides an ideal model system for examining lingering anti-brood parasitism defenses.

In this study, we introduce and deploy 3D printed facsimiles of adults and eggs of putative brood parasites for examining lingering defenses in the Australian reed warbler and use these to help identify the warbler's ghost of parasitism past. This method allows us to examine various defensive responses without using costly, fragile, and difficult to obtain taxidermy specimens (Aragon et al. 1999; Welbergen and Davies 2008; Hauber et al. 2015; Tryjanowski et al. 2018). First, we confirm whether egg discrimination ability observed by Welbergen et al. (2001) does, in fact, occur elsewhere across Australia's eastern reed warbler population. Second, we conduct an

"cuckoo at the nest" experiment using 3D printed models of various cuckoo species to (1) determine whether the reed warbler has frontline defenses indicative of a history brood parasitism and (2) to identify the ghost of parasitism past from an array of candidate species. We expected that if the reed warbler has indeed coevolved with a brood parasite in the past, and won, then it should exhibit defenses that are (1) appropriate in the general context of brood parasitism, (2) found at multiple stages of the host's nesting cycle, and (3) specific to facsimile models of its former parasite.

Materials and Methods

Study species
The AAustralian reed warbler (ARW) is a small passerine songbird that closely resembles the old-world reed warblers of Eurasia. It is distributed along the seaboard of Australia. The incubation period for the Australian reed warbler is 14 days, and eggs (average clutch size 2.8) are usually laid every day but with the occasional 1-day laying gap (Welbergen et al. 2001; Berg et al. 2006).

The studied population overlaps that of all Australasian cuckoo species except the little bronze-cuckoo *Chalcites minutillus*. These species include the oriental cuckoo *Cuculus saturatus*, pallid cuckoo *Heteroscenes pallidus*, fan-tailed cuckoo *Cacomantis flabelliformis* (morphologically similar to *C. variolosus*), Horsfield's bronze-cuckoo *Chalcites basalis*, and the shining bronze-cuckoo *Chalcites lucidis*, channel-billed-cuckoo *Scythrops novaehollendiae*, eastern koel *Eudynamis orientalis*, chestnut-breasted-cuckoo *Cacomantis casteneiventris*, and the black eared-cuckoo *Chalcites osculans* (http://www.birdlife.org.au/conservation/science/taxonomy). Of these species, 4 are known to very occasionally parasitize the Australian reed warbler (fan-tailed cuckoo: 2 records; pallid cuckoo: 7 records; Horsfield's bronze-cuckoo: 2 records; and shining bronze-cuckoo: 2 records from 133 breeding seasons). Previous studies following the breeding of Australian reed warblers found no evidence of conspecific parasitism (Brooker and Brooker 1989; Berg 1998; Welbergen et al. 2001), and in their study, Welbergen et al. (2001) failed to find evidence of conspecific brood parasitism in this species (Berg 1998). Apart from these rare reports of "incidental" parasitism, there are no known cases of recurrent or frequent brood parasitism in this species (Brown et al. 1990; Welbergen et al. 2001). In all cases, cuckoo eggs but not chicks have been observed, suggesting that such incidental parasitism is rarely, if ever, successful.

Study sites
This study was conducted in the outer western suburbs of Sydney, Australia, covering 12 sites consisting of wetland shrubs and rushes, situated along the Nepean River basin between Emu Heights (150°38′56″ E, 33°43′54″ S) and Pitt Town (150°85′46″ E, 33°58′94″ S). The fieldwork took place between 18 August 2015 and 1 February 2016, coinciding with the reed warbler breeding season (September to January) (Welbergen et al. 2001). Australian reed warblers were observed at the field sites from 12 September 2015, and the first nest was discovered 2 weeks later on 26 September 2015 at Pitt Town Lagoon.

To find nests, we first located reed warbler territories using responses of territory holders to playback of reed warbler calls from a wireless speaker (JBL GO; *HARMON-INTERNATIONAL INDUSTRIES, CHINA*), and looked for nests in these territories, by "cold-searching" the reeds *Phragmites australis*, a habitat in this area associated with extensive wetland and few trees, or rushes *Typha orientalis* that were associated with smaller waterbodies surrounded by trees. Reeds, in particular, are subject to damage as one moves through them, so we took great care to minimize damaging the vegetation (Davies and Brooke 1988; Welbergen et al. 2001). Over the season, we found 173 Australian reed warbler nests.

Nesting locations were recorded with a Global positioning system (GPS) application (*GPS Essentials Android*) (accuracy ±3–6 m) and tagged using pink construction tape. A tag was attached above a nest, tied to a prominent reed stem, and again below the nests with a nest ID label providing a means of identification.

Egg rejection experiments
To confirm whether egg rejection behavior observed by Welbergen et al. (2001) in a reed warbler population in Victoria did, in fact, occur in our New South Wales (NSW) study population, we conducted a very simple experiment by presenting 1 artificial egg from 1 of the 5 species of brood parasite to 5 reed warbler nests. These experiments broadly replicated the methods from Welbergen et al. (2001); however, instead of using conspecific and wax-based eggs, we used 3D-modeled eggs from the oriental cuckoo, pallid cuckoo, fan-tailed cuckoo, Horsfield's bronze-cuckoo, and the shining bronze-cuckoo. The ($n = 5$) eggs were sourced and scanned from the Australian Museum Collection, Sydney, to produce generic eggs for this experiment. These models were then printed using a 3D printer and filled with natural beeswax to replicate the appropriate mass of the living specimens.

The method we used to print the eggs is similar to methods used by Igic et al. (2015); however, we improved the process by coating our eggs with a layer of natural beeswax, prior to painting with an off white monochromatic matte layer, a process that allows peck marks associated with egg rejection events to be recorded. Each of the chosen eggs was roughly similar in size and weight to the eggs (2.0 g ± 10%) of the Australian reed warblers own (see Welbergen et al. 2001). We then deposited the selected eggs in the nests of reed warblers during the laying period, or no >2 days after the clutch had been completed. If there had been 3 days since the addition of an egg to the nest, the clutch was deemed complete and checked for signs of egg warmth, indicating that incubation had commenced. Egg rejection experiments took place after clutch completion, during the first 4 days of incubation, as at least 2 days were required for the experiment. We then determined the reed warbler's subsequent acceptance or rejection of artificially placed eggs. Simultaneously, as a control, we recorded any evidence of rejection and ejection behavior in a further 20 nests that were treated identically to the experimental nests except that no artificial eggs were added.

Cuckoo at the nest experiments
To test the Australian reed warbler's "frontline" defenses to artificial parasitism, we manufactured 2 replicate 3D-printed taxidermy facsimiles each of 5 species of Australian cuckoo as parasite stimuli, and a dove as a control (Sup). These 3D-printed "mounts" allowed us to examine defensive behaviors in response to a variety of potential brood parasites and a control, without risking damage to costly, rare, and fragile taxidermy specimens.

To create the mounts, taxidermy specimens (sourced from the Australian Museum avian collection) were scanned (Figure 1) using a hand-held Sense-3D scanner (3DSYSTEMS, Rock Hill, SC, USA). Mounts were printed using Acrylonitrile Butadiene Styrene plastic. Mount pairs were fundamentally the same, although some features, such as body (tail feather length and width) and beak length

Figure 1. 2D stills of 3D scans "at nest pose" includes all species and their eggs used in experiment—Oriental cuckoo *C. saturatus*, Pallid cuckoo *H. pallidus*, fan-tailed cuckoo *C. flabieloformis*, Peaceful dove *G. straita* (control), Horsfield's bronze-cuckoo *C. basalis*, shining bronze cuckoo *C. lucidis*.

were manipulated in line with natural variability, and some mounts were mirrored in 3D space before printing, so the printed replicates were slightly different from one another. These subtle changes were introduced to minimize the potential for pseudo-replication. In behavioral studies, balsawood mounts are common practice, and have been shown to elicit no differences in responses when compared with taxidermy mounts (Welbergen and Davies 2008, 2012; Tryjanowski et al. 2018).

Each 3D-printed mount was painted and fitted with a mounting shaft made from flexible fencing wire (22 cm long), enabling them to be readily mountable and interchangeable across trials. Paint colors were matched approximately to the feather pigments by using a pairwise technique, whereby the feather and paint pigments were photographed using a Nikon D70 camera, an unfiltered Nikkor 105 lens, and Rosco perimicolor UV pass filter under a high-intensity discharge lamp (xenon bulb). Reflectance and luminance values were identified by using the micaToolbox (Vers 1) plugin which was used as part of the ImageJ software (Troscianko and Stevens 2015). This process produced high, medium, and low, UVa and UVb layers which were linearly processed, resulting in calibrated photographs able to be reliably analyzed for reflectance color and luminance values. This enabled color matching across the visual spectrum (360–700 nm), encompassing the wider spectral sensitivity shown in birds (Huth and Burkhardt 1972). Mounts were positioned on mounting poles (bamboo stakes) that were placed at the nest the day preceding the trial. Mounts were positioned ∼5 cm from each nest ($n = 35$) so that they emulated an attempted parasitic laying effort (Sup) and maximized the potential mobbing response (Welbergen and Davies 2012). These procedures matched established protocols set out in experimental parasitism studies on *Acrocephalus scirpaceus* (Welbergen and Davies 2008, 2009; Davies and Welbergen 2009; Davies 2011; Welbergen and Davies 2011; Welbergen and Davies 2012).

Host responses to the mounts were recorded using a GoPro-Hd (x1) or an AEE A60 Magicam (×4) high-definition, wide-angle, waterproof, and action camera. A single camera was attached to a bamboo stake that had been secured to surrounding reeds, using twine to prevent camera movement during the experiment. Cameras were installed ∼1–1.5 m from the nest, and 1 day prior to the experiment so that the warblers would habituate to the camera's presence. A viewing platform (a ladder) was positioned at a distance of ∼15 m from the nest, so that observations with line-of-sight to the nests,

along with the neighbors approach trajectories, could be made (Davies and Welbergen 2009).

Experiments usually began in the morning. Upon approach nest owners left the area. A 5-min timer was activated as soon as a nest owner was observed returning within 1 m of the nest. The presence of the first returning bird was determined through the characteristic shuffling of the reed as the bird hopped on to and moved up and down the reed as they returned to the nest. To facilitate these observations, experiments were conducted only when local wind force was less than 4–5 Beaufort. Each trial was terminated 5 min after the first approach; this was done to standardize the duration of exposure for the birds among the trials. If no approach was recorded within the first 15 min, the trial was terminated (27 trials were terminated in this fashion). Thus, the total duration of a trial never exceeded 20 min (see also Welbergen and Davies 2012), which was to minimize the risks that the focal birds became habituated or sensitized to the mounts.

One mount specimen was presented per trial, and up to 6 mount specimens were presented at each focal nest, so that each species of cuckoo, and the control, were presented at each nest using the same procedure. Two sets of 6 mounts (A, 1–6 and B, 1–6) were alternatively deployed between nests. Presentations of the 6 mount specimens were balanced following a Latin-square design to compensate for potential order effects (e.g., Welbergen and Davies 2012).

The mount species included endemic brood parasite species the oriental cuckoo *C. saturatus*, pallid cuckoo *H. pallidus*, fan-tailed cuckoo *C. flabelliformis* (morphologically similar to *C. variolosus*), Horsfield's bronze-cuckoo *C. basalis*, and shining bronze-cuckoo *C. lucidis*. Also included as a control was the peaceful dove *Geopelia striata*, chosen for its intermediate size, color, and its nonthreatening morphology (Brooker and Brooker 1989; Simpson et al. 1996; Welbergen et al. 2001) and plumage (Welbergen and Davies 2011). Except for the oriental cuckoo *C. saturatus*, the selection of each potential parasite species was based on the following criteria: they overlap in distribution with the warbler; share an insectivorous diet; and have an appropriate body size and egg size for parasitism of the warbler (Brooker and Brooker 1989). The oriental cuckoo is present, but does not breed in Australia, and was included in the study because it is known to parasitize congeneric reed warblers (*Acrocephalus* spp.) across Asia and might have bred in Australia in the past. The diversity of species provides a morphologically distinct range, with sizes ranging from 16 to 33 cm long and varying coloration, suggesting that each species is distinct enough not to be easily confused with one another. Though differing in several plumage features, the Horsfield's and shining bronze-cuckoos are morphologically similar and have been shown to elicit similar reactions from their hosts (Payne et al. 1985).

We carried out experiments at 35 nests. By design, each replicate was made up of 6 trials; however, some nests ($N = 7$) were not exposed to all 6 mounts due to predation ($N = 2$), hatching ($N = 1$), and nest damage (floods) followed by abandonment ($N = 4$). This meant that the total number of successful trials across nests was 183. The complete set of 6 trials was conducted over 2–6 days, during daylight hours, allowing for no >3 trials per nest, per day. Trials at a focal nest were separated by a respite period of at least 2 h, allowing for reed warbler responses to return to the baseline level (Welbergen and Davies 2009).

Video recordings of nest defenses were scored and analyzed using Garageband (version 10, Mac) for file conversion (video to audio), Audacity (version 2.1.0, Mac) for visualization of sound, and VLC (version 2.2.2, Mac) for video playback.
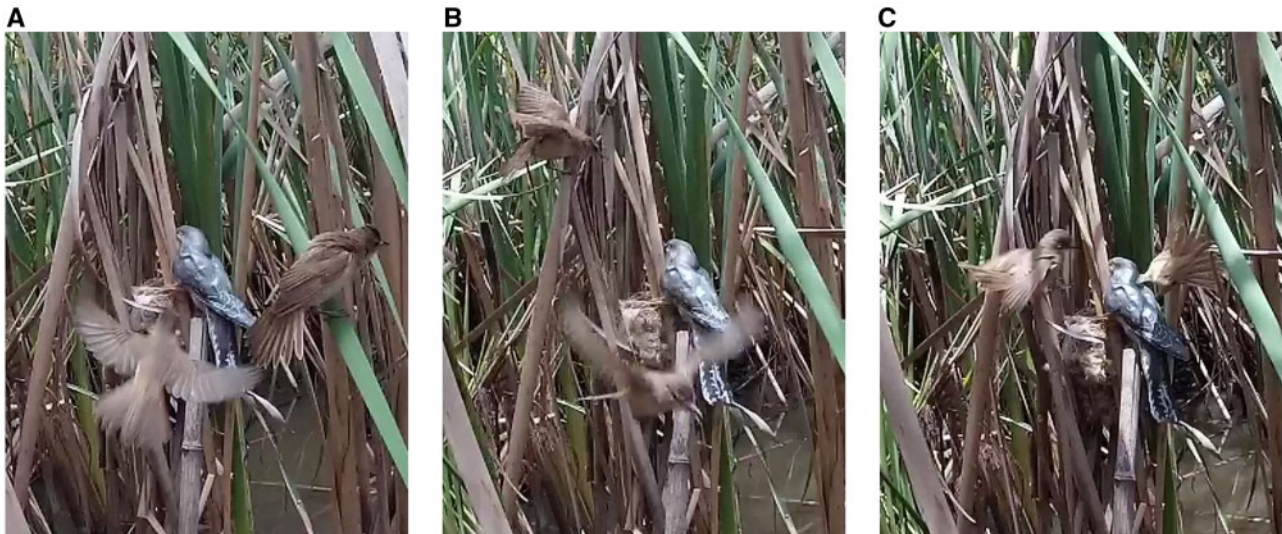
Figure 2. A pair of Australian reed warblers directly attacking the mount in their rush habitat.

The frontline responses that were assessed included: "mobbing behavior" which involved calls, mandible snaps, mobbing numbers, threat postures, and direct physical attack (Welbergen and Davies 2008) close to the nest intruder (Figure 2); "alarm signaling" that includes alarm calls at some distance from the nest invader; and "nest-sitting," a passive defense (not previously described in this species) in which the bird sits on the clutch, preventing access to the nest intruder (Gill and Sealy 2004).

From the video recordings we scored the following nest defense-associated audible signals and behaviors: "bill snaps" are the percussive clicking sound of the beak closing with force, sounding similar to a small twig being snapped in two (Figure 3A); "churr" comprises a repetitive chatter vocalization, sounding like an old-fashioned machine gun from a distance (Figure 3B); "krek" is a short alert chirping alarm call (Figure 3C); "alarm" is similar to the krek call but sharper and more urgent "seet" like note (Figure 3D); "rasp" is an often-repetitive harsh vocalization that is a more aggressive form of the churr call where each vocalization is extended and emphasized in volume and duration (Figure 3E); "song" is lyrical, multi-noted and has a complex delivery of notes (Figure 3F). "Physical attack" involved the reed warbler making beak contact with the mount (Figure 2), and nest sitting occurs when the host sits on the nest whilst the mount is at the nest.

Statistical analysis

All analysis was carried out using the R environment for statistical computing, version 3.4. 1. (R Core Team 2017). Tests were 2-tailed, with a significance threshold set at 0.05, and means were conveyed using ±standard deviation (SD) where appropriate. For the experiment examining egg stage defenses, we used a Fisher's exact test to evaluate the differences in ejection rates between the artificially parasitized ($N = 5$) and control ($N = 20$) nests. For the experiment examining frontline defenses, we first used a principal component analysis (PCA) on 9 variables—vocalizations ("krek," "churr," "chatter," "rasps," bill snaps, song, and alarm), and actions—"direct attack" and "nest sitting" to reduce the number of predictor variables, and identify correlated defensive behaviors (Shlens 2003) (see Table 1). PCs with Eigenvalues of >1, or very close to, were retained (see Table 1). In the analysis (PC 1–3 were held), and those

that did not meet the loading criterion were to be abandoned. We then used general linear mixed models, R package (lmerModLmerTest-Lme4) with "nest ID" as a random factor to determine the effects of "mount species," "trial order," "reed type" (fixed factors), on PC 1–3 from the PCA (above). All 3 statistical models were checked for violations of model assumptions, and in all cases, the residuals were normally distributed.

Results

Experiment 1: egg discrimination

The egg rejection rate from experimental nests was significantly higher than the natural rejection rate (if any) from unparasitized nests (5/5 vs. 0/20; Fisher's exact test: $P = 0.002$). All artificial eggs placed in experimental nests were rejected by being ejected from the nest within 48 h, so there were no acceptance errors. Among experimental nests, only artificial eggs were rejected and no host eggs (5/5 artificial eggs vs. 0/15 host eggs, Fisher's exact test: $P < 0.005$), so there were no ejection errors.

Experiment 2: cuckoo at the nest

Defensive patterns

The first 3 components of the PCA had Eigenvalues >1 and explained 72.3% of the total variance (Table 1). In sum, PC1 (38.3% of the overall variance), had the highest loadings from the churr and rasp calls, bill snaps, and direct attack variables, as well number of warblers present during mobbing events, and so was termed "*mobbing behavior.*" PC2 (23% of the variance) had the highest loadings from the krek, song, and alarm calls variables, and so was termed "*alarm signaling.*" PC3 (11% of the variance) had the highest loading from nest sitting, and so was termed "*nest sitting.*"

Responses according to mount species

Over the course of 183 trials, from $n = 35$ nests, all 6 mount-species were mobbed, and warblers exhibited some form of nest defense in 94% of trials. Nest owners approached the mount on average 29.1 ± 36.03 cm, irrespective of the species of the model. Overall, there was a marginally significant difference found between the reed
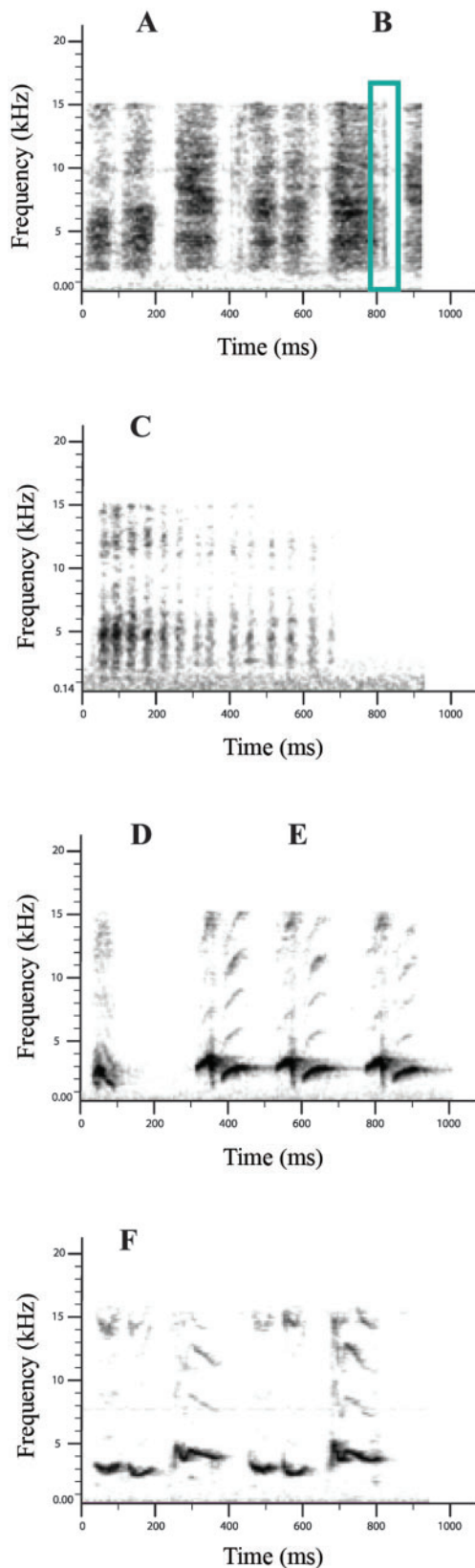
Figure 3. A visual representation of the analysis showing the types of calls identified and counted. (A = bill snap, B = churr, C = krek call, D = alarm call, E = rasps, F = song).

41.7 ± 40.06 cm and rush defense distances 18.8 ± 28.64 cm, $t = 1.98$, $df = 33$, $P = 0.056$, though the differences were significant for the aggressive behaviors (see ahead). Two particular defensive behaviors committed some birds to a closer approach: "nest sitting" (20% of trials) occurred between 5 and 0 cm from the mount, and mobbing or "direct attack" (15.4% of trials), which involved the birds making contact (often repeated) with the mount (aggressively pecking the skull and torso).

Mobbing behavior (which involved the hosts directly engaging with the mount through physical or audible cues [attack, bill snaps, churr, and rasp] that appeared to signal proactive aggression toward the mount) occurred at 51% of trials, and alarm signaling (involved only vocal cues [song, alarm, and krek] that appeared to be a nonaggressive) occurred at 84% of trials. Mobbing behavior was accompanied by alarm signals in 41% of trials. Although some nests had up to 6 birds mobbing the mount simultaneously, the average number of nest defenders identified in these experiments was 1.55 ± 0.92 per trial.

Mobbing behavior was not significantly affected by mount species, but there were significant effects of order, and of vegetation type, with birds nesting in *T. orientalis* habitat being more aggressive than those nesting in *P. australis* habitat (Table 2). Alarm signaling was also not significantly affected by mount species, nor were there any significant order or vegetation type effects (Table 3). Finally, nest sitting was also not significantly affected by mount species nor by the other covariates (see Table 4). Thus, the reed warblers appeared to deploy their multimoded defensive strategies irrespective of the type of mount presented at the nest and were equally aggressive to both the parasitic and nonparasitic mounts (see Figure 4).

Post-trial nest visits occurred at 20 of the 35 experimental nests, and in all but 2 of those 20 nests, healthy chicks were observed. Therefore, nest desertion does not appear to be a common response to parasitic nest intruders in this species.

Discussion

This study, the first to present 3D-printed models of brood parasites at host nests, showed that despite the absence of a current brood parasite, the Australian reed warbler readily engages in frontline and egg-stage defenses that are considered adaptive specifically in the context of brood parasitism (i.e., egg rejection and nest sitting). Along with behaviors considered to be a generalized defense against brood parasites and other threats in general (i.e., mobbing). However, no species-specific response was found. We also conclude that 3D facsimiles are a potentially suitable alternative to taxidermy and balsawood models, providing researchers with a readily available source of mounts that are reproducible, manipulable in a controlled manner, inexpensive, resilient, and effective.

This study relied on the novel use of 3D printed models of eggs and adult birds presented at nests. The functionality of 3D-printed eggs in experimental parasitism experiments had been previously established (Igic et al. 2015). We employed the use of 3D-printed models (adult birds) for the first time in "cuckoo at the nest" experiments and therefore represents an important methodological advance in behavioral research that relies on artificial stimuli as part of the experimentation process (Hauber et al. 2015). The strong reed warbler responses observed in reaction to the mounts lead us to conclude that 3D mounts were a suitable alternative to both balsa wood

Table 1. Principal component loadings for quantified variables and total variance explained by each component that resulted from analysis of defensive type variables including cumulative proportion of variance explained by each component

Loadings	PC.1	PC.2	PC.3	PC.4	PC.5	PC.6	PC.7	PC.8	PC.9
Krek	−0.114	−0.564	0.144	0.204	−0.171	0.468	−0.475	0.363	–
Churr	−0.406	–	−0.167	−0.631	−0.133	0.128	0.599	–	–
Rasp	−0.411	0.164	0.111	0.537	0.221	0.190	0.441	0.229	0.415
Bill snaps	−0.493	0.158	–	0.206	0.168	−0.107	–	–	−0.798
Direct attack	−0.451	0.117	–	–	–	−0.366	−0.619	−0.261	0.431
Nest sitting	0.161	–	0.924	−0.142	−0.233		0.193	–	–
Song	–	−0.555	−0.113	0.270	−0.282	−0.675	0.248	–	–
Alarm calls	−0.114	−0.529	–	−0.296	0.743	0.152	−0.200	–	–

Table 2. Results from the GLMM evaluating the influence of the nest invader species, the reed type, and order of mount presentation on the mobbing behavior

Fixed effects	Effect size	±SE	t-value	$Pr(>|t|)$
Intercept	0.605	0.424	1.428	0.160
Fan tailed	0.080	0.188	0.423	0.673
Shining	−0.076	0.189	−0.400	0.690
Oriental	−0.156	0.187	−0.833	0.406
Pallid	−0.079	0.127	−0.424	0.672
Peaceful dove	0.159	0.186	0.856	0.393
Reed type	−1.504	0.531	−2.834	0.008**
Order	0.086	0.032	2.654	0.009**
Random effects		variance	SD	
Nest ID		2.313	1.521	** $P \leq 0.01$

Table 3. Results from the GLMM evaluating the influence of the nest invader species, the reed type, and order of mount presentation on the alarm signaling

Fixed effects	Effect size	±SE	t-value	$Pr(>|t|)$
Intercept	−0.286	2.109	−0.136	0.892
Fan tailed	−0.368	0.930	−0.395	0.693
Shining	−0.379	0.325	−1.165	0.246
Oriental	−1.097	2.191	−0.501	0.617
Pallid	−0.897	1.935	−0.463	0.644
Peaceful dove	−0.325	0.841	−0.386	0.700
Reed type	−0.294	0.344	−0.855	0.399
Order	−0.019	0.052	−0.374	0.709
Random effects		Variance	SD	
Nest ID		1.513	1.23	

Table 4. Results from the GLMM evaluating the nest invader species, the reed type, and order of mount presentation on the nest sitting response

Fixed effects	Effect size	±SE	t-value	$Pr(>|t|)$
Intercept	0.309	1.310	0.236	0.814
Fan tailed	0.065	0.573	0.113	0.910
Shining	0. 007	0.197	0.038	0.970
Oriental	0. 4560	1.355	0.336	0.737
Palid	0.318	1.197	0.265	0.791
Peaceful dove	0.128	0.519	0.247	0.805
Reed type	0.036	0.274	0.133	0.895
Order	0.040	0.031	1.288	0.200
Random effects		Variance	SD	
Nest ID		0.535	0.732	

models and expensive and rare and fragile taxidermy mounts. Previous studies support the claim that facsimile mounts made out of balsa wood are mobbed at the same rates as taxidermy specimens (Welbergen and Davies 2012). The 3D-printed mounts also provide extra benefits—they are sturdy, repairable, waterproof, and can be manipulated quantitatively or qualitatively and printed in various controllable and reproducible ways. Furthermore, access to 3D files allows for studies to be effectively and efficiently replicated.

In our simple confirmatory experiment, we found clear evidence of egg rejection, with $n = 5$, or, 100% of artificial eggs rejected, despite the lack of brood parasitism observed in our local population (this study) and the species as a whole (Brooker and Brooker 1989). This behavior cannot be explained by the species' usual brooding behavior, as no host eggs were rejected from the unparasitized nests, there was also no evidence for recognition errors in this population. These results thus confirmed that, like the Victorian population reported in Welbergen et al. (2001), reed warblers in our study population also exhibited egg discrimination behavior. As egg discrimination is an adaptation that evolves specifically in the context of brood parasitism, this provides clear indications of lingering egg-stage defenses in this population. Similar results were obtained from a Victorian population where ~81% of artificial eggs were rejected (Welbergen et al. 2001), but not from a Western Australian population (Brown et al. 1990) where rejection rates were significantly lower than those observed in Victoria (Welbergen et al. 2001). Variable egg rejection rates among host populations are generally thought to reflect geographic variation in selection pressures from the parasite, albeit modified by individual variation within populations (Soler et al. 1999; Lindholm and Thomas 2000; Stokke et al. 2008; Welbergen and Davies 2012). Consistent with the "geographic mosaic theory of coevolution" (Thompson 1994), the difference in the rejection rates between the Victorian and Western Australian reed warbler populations are likewise thought to have evolved due to a deep east-west discontinuity in reed warbler's distribution (Welbergen et al. 2001), separating subspecies *A. australis australis* in the east from *A. australis gouldi* in the west. Therefore, we speculate that the results observed in our NSW population, another eastern regional group of the *A. australis australis* subspecies, further suggest that the Australian reed warbler's lingering egg-stage defenses are due to parasitism that occurred sometime after subspeciation, thought to have occurred sometime within the last 0.6 myr (Jetz et al. 2012).

In our principal experiment, all brood parasite and control facsimiles were confronted by one or more reed warblers (up to 6) at most nests. Three distinct defensive patterns were found at the frontline; "mobbing behavior," "alarm signaling," and "nest sitting," each consistent with defensive behaviors observed in a variety of currently parasitized hosts (Gill and Sealy 2004; Welbergen and
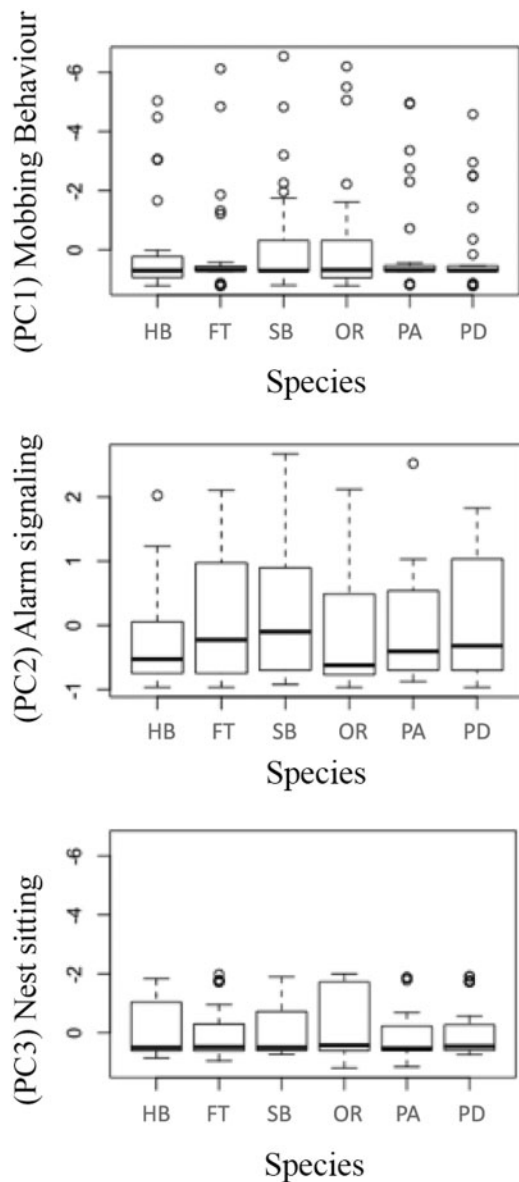
Figure 4. Boxplot of raw PCA results evaluating the relationship between nest invader species and strength of defense. Horsfield's bronze-cuckoo (HB), fan-tailed cuckoo (FT), Shining bronze Cuckoo (SB), Oriental cuckoo (OR), Pallid cuckoo (PA), Peaceful dove (PD). The box shows the interquartile range, whiskers above and below show the 10th and 90th percentiles. Circles show outliers.

Davies 2008; Rands 2012). Additionally, a clear effect of habitat on defensive behavior was found; owners of nests in particular habitat were significantly more aggressive than owners of nests in dense habitat, suggesting that the Australian reed warbler varies its mobbing strategies in response to perceived but indirect threat cues in ways similar to related, parasitized *Acrocephalus* species (Duckworth 1991; Welbergen and Davies 2012).

A priori, however, it is not clear if the observed defenses are specific anti-brood parasite adaptations because frontline exposure to various nest invader types such as predators and nest predators is

likely. Parsing out the evolved origin of the reed warbler behavior thus requires a closer look at the behavior's threat-specific utility. Of the 3 observed defensive strategies, alarm calling and mobbing represent the most equivocal, because these are seldom a specific response to brood parasitism (but see Feeney and Langmore 2013), and are adaptive in a multitude of contexts (Haff and Magrath 2013; Mahr et al. 2015; Barati and McDonald 2017). Yet, there is substantial evidence in reed warblers and other hosts that alarm calls and associated mobbing behaviors are ultimately adaptive and reduce exploitation in the context of brood parasitism (Welbergen and Davies 2008, 2009; Langmore et al. 2009; Rands 2012; Thorogood and Davies 2016). However, given the dangers of nest defense at the frontline, it makes functional sense that hosts should be reluctant to engage in behaviors, such as nest sitting, that risk close interactions with predators (Campobello and Sealy 2018), and should, therefore, be sensitive to the risks posed by various enemies (Welbergen and Davies 2009). Consequently, this form of frontline defense can only be adaptive in the context of parasitism, and accordingly nest sitting has only been reported in the context of brood parasitism (e.g., Gill and Sealy 2004; Canestrari et al. 2009; Rands 2012). This reasoning would particularly apply in the context of this study where the most common nest predators, such as the pied currawong *Streptera gracilina* and the grey butcherbird *Cracitus torquatus*, also predate smaller birds. But also, because multiple cuckoos presented in this study appear to have evolved some aspects of predator mimicry. Furthermore, nest-sitting reed warblers spread their bodies over the nest opening and remain continually focused on the intruder, a behavior that differs, appreciably, from normal brooding behavior. At no time did a sitting warbler leave the nest before the researcher had approached the nest to remove the facsimile (*pers obs*), which demonstrates the reed warbler was vigilant and determines nest invaders as a threat. Nest sitting, as observed in this study, consequently, is expected to have evolved exclusively in the context of nest invaders that pose no threat to the adult reed warbler (Montgomerie and Weatherhead 1988), therefore, as an anti-parasite defense. This reasoning is supported by prior research and suggests that "nest sitting" (Gill and Sealy 2004; Rands 2012) is a defensive strategy specific to brood parasites, sensitive to the particular costs imposed by brood parasites and predators. Although we cannot exclude the possibility that the nest-sitting birds find the mounts unconvincing, we suggest that it is unlikely, given the strong responses of the warblers in this study, and the success of similar methodologies in comparable systems (see Welbergen and Davies 2008, 2009).

These findings, in association with egg rejection in multiple reed warbler populations (Welbergen et al. 2001) support the argument (Aragon et al. 1999; Medina and Langmore 2015b; Soler 2014) that suitable hosts that are not currently parasitized are likely to have "won" the arms race through highly effective defenses against brood parasitism. They furthermore lend support to the "defense-in-depth" hypothesis (Welbergen and Davies 2009), suggesting that effective anti-parasite defenses may arise at each of the stages through prolonged and incremental selection pressures, and simultaneously at multiple stages of the nesting cycle. Though defenses at the nestling stage are yet to be formally investigated, we suggest that the Australian reed warbler has retained, in the absence of selection, a generalized defensive integrity that currently renders the species immune to naïve brood parasitism. These defenses provide good evidence that the Australian reed warbler, like its Eurasian congeners, exhibits multiple lingering defensive lines against would-be brood parasite, and implies that the species is relatively immune to new

parasitism, at least into the near-evolutionary future. This is consistent with the single trajectory model of brood parasite–host coevolution that suggests that brood parasites ratchet through host populations discarding them when the cost of parasitism becomes prohibitive (Peer et al. 2011).

We found no evidence that the Australian reed warbler's lingering defenses, at the frontline (mobbing behavior, alarm signaling, and nest sitting), are responses tuned to specific nest intruder cues (i.e., one of the species of cuckoo, or the dove control). Recently, learning has emerged as an important component of host defenses against brood parasitism (Campobello and Sealy 2018) at the frontline (Feeney et al. 2012; Feeney and Langmore 2013), egg stage (Moskát et al. 2014), and chick stage (Langmore et al. 2009) of the host nesting cycle. However, in the absence of biological parasitism, there is no opportunity to learn an anti-parasitism response, and indeed, even when the opportunities for learning are few (incidental parasitism for example), host species tend to fail to discriminate between known brood parasites and controls—which has been described as a "cultural loss" of enemy discrimination at the frontline (Lahti 2006; Antonov et al. 2007; Hale and Briskie 2007). Thus, although the presently nonparasitized reed warbler readily engages in frontline and egg-stage defenses that are considered adaptive generally, and in the case of nest sitting, specifically, in the context of brood parasitism, a lack of learning opportunities would explain the species inability to identify its brood parasite from the past, or distinguish between cuckoos and the control. Another example of a failure to discriminate between mounts of a parasite, the common cuckoo *C. canorus* and a control, the feral pigeon *Columbia livia*, at the frontline was recorded in populations of the great reed warbler *Acrocepholaus arundinaceaus*—where the parasitism rate was low (*Czech Republic*), these results were compared with other host populations (*Hungary*) that have a high rate of brood parasitism. Subsequently, the study showed significant levels of discrimination between mounts (Honza et al. 2006). In each of these cases, the inability to differentiate between the cuckoos and the controls would rely on previous experience at the nest. This corroborates our interpretation that Australian reed warblers are responding to each of the mounts contextually, as novel brood parasites (also see Welbergen and Davies 2012). Therefore, the role of learning in Australian reed warbler defenses should be tested in the future.

Our study has shown that by recreating putative bygone brood parasite–host interactions, using artificial parasitism experiments with 3D-printed models, lingering behaviors consistent with antiparasite defenses can be detected experimentally. More such experiments on a broader range of species are needed to fully appreciate the importance of lingering frontline anti-brood parasite defenses in protecting prospective hosts from future parasitism. We also conclude that 3D facsimiles are a potentially suitable alternative to taxidermy and balsawood models, providing researchers with a readily available source of mounts that are reproducible, manipulable in a controlled manner, inexpensive, resilient, and effective; although, future research is needed to compare the responses of nest owners to 3D models to those taxidermy models and real nest enemies. Our approach also provides exciting opportunities for "behavioral archeological" research, particularly in systems with experience-independent anti-parasitism defenses where these experiments hold the most promise for unearthing the true identities of the "ghosts of parasitism past."

Author Contributions

M.H.J.C. and J.A.W. formulated the ideas and developed methodology; M.H.J.C. conducted fieldwork; M.H.J.C. performed analyses; and M.H.J.C. and J.A.W. wrote the manuscript.

Data Accessibility Statement

Analyses reported in this article can be reproduced using the data provided by Matt Chaumont (2020).

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