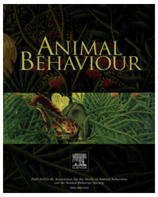
Animal Behaviour 122 (2016) 177–181

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav



Egg recognition as antiparasitism defence in hosts does not select for laying of matching eggs in parasitic cuckoos

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ARTICLE INFO

Article history:
Received 25 June 2016
Initial acceptance 27 July 2016
Final acceptance 15 September 2016

MS. number: 16-00557R

Keywords:
Acrocephalus orientalis
avian brood parasitism
Cuculus canorus
egg mimicry
natural selection

Recent studies have suggested that parasitic cuckoos have evolved laying behaviour resulting in matching of host and cuckoo eggs by choosing to lay eggs in host nests with host eggs that match the cuckoo eggs as an adaptation against egg recognition by the hosts. However, previous studies provided weak and indirect evidence with mixed results, leaving this question unresolved. Here, for the first time, we developed a robust methodology to provide unambiguous evidence that egg recognition in the host does not select for optimal egg matching during laying by the cuckoo. By using experiments that attracted parasitism, we showed that cuckoos, *Cuculus canorus*, indiscriminately laid eggs in oriental reed warbler, *Acrocephalus orientalis*, host nests containing real host eggs, egg-shaped models, stick models or coin models without any preference. Furthermore, cuckoos only selected to lay their eggs in nests with active hosts. These experiments provide evidence of cuckoos being indiscriminate in their choice of host nests, implying that coevolution of the egg phenotype of host and cuckoo eggs must have arisen from mechanisms other than matching of host eggs and those of the parasite.

Natural selection is a driving force in biological evolution (Darwin, 1859). Biotic sources of natural selection are plentiful, especially in biological interactions. For example, parasites impose costs on their hosts during parasitism that select for better host defences, which in turn select for counter-defences in parasites (Thomas, Guégan, & Renaud, 2009). Therefore, both parasites and hosts act as agents of natural selection and drive mutual evolution in both parties during their interaction.

In birds and a few other groups, some taxa do not care for their own offspring, but transfer the reproductive costs to another species (Soler, 2014). These obligate brood parasites lay their eggs in other species' nests. As a model system for the study of coevolution, cuckoos are the most well-known parasitic birds that cheat their hosts by laying eggs with a mimetic appearance (Davies, 2000).

At the beginning of the host–parasite interaction, cuckoos do not produce mimetic eggs because naïve hosts do not possess any capacity of egg recognition (Davies, 2011; Yang, Wang, Cheng et al., 2015). Subsequently, the fate of cuckoo eggs depends on their similarity to host eggs when hosts evolve egg recognition, and such cuckoo egg mimicry increases with escalation of the egg recognition capacity in hosts (Soler, 2014; Yang, Su, Liang, & Møller, 2015). Because egg mimicry increases the success of cuckoo eggs, it has been hypothesized that cuckoos would choose to lay eggs in host nests with eggs similar to their own (Avilés et al., 2006; Cherry, Bennett, & Moskát, 2007; Honza, Šulc, Jelínek, Požgayová, & Procházka, 2014).

Empirical tests are consistent with the hypothesis that egg recognition as antiparasitism defence in hosts selects for egg mimicry in the cuckoo, thereby promoting coevolution (Davies, 2000; Moksnes, Røskaft, & Braa, 1991; Yang et al., 2010; Yang, Wang et al., 2014). However, whether cuckoos choose hosts whose eggs match their own still remains uncertain with results being mixed and the evidence overall being inconsistent. Previous studies generally tested this hypothesis by comparing egg matching between cuckoo and host eggs in parasitized nests and nearby nests that were not parasitized. The latter nests were assumed to be found by cuckoos, but not used for parasitism owing to poor egg matching compared to the nests that were chosen (Antonov et al., 2012; Avilés et al., 2006; Cherry et al., 2007; Honza et al., 2014; Yang, Wang, Liang, & Møller, 2016). However, the results were mixed: some supported the hypothesis (Avilés et al., 2006; Cherry et al., 2007; Honza et al., 2014) but others did not (Antonov et al.,

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http://dx.doi.org/10.1016/j.anbehav.2016.10.018

2012; Yang, Wang et al., 2016). Furthermore, all previous evidence is weak because it is indirect and based on several assumptions that have not been proven (Yang, Wang et al., 2016).

Here we developed a much more robust methodology to test this hypothesis by setting up combinations of host (Oriental reed warbler, *Acrocephalus orientalis*) nests differing in degree of egg matching to directly attract parasitism from common cuckoos, *Cuculus canorus*. This experimental design provided cuckoos with clustered nests that contained different egg/object phenotypes for their selection during parasitism, and thus we could directly investigate the preferences of cuckoos during parasitism. Because egg recognition by hosts is a necessary condition that may drive the evolution of optimal laying behaviour in cuckoos, we also conducted a parasitism experiment to assess the egg recognition capacity of our study population, although previous studies have shown that this is strong in this host species (Li, Zhang, Grim, Liang, & Stokke, 2016; Lotem, Nakamura, & Zahavi, 1992, 1995).

If laying behaviour is not under selection from egg recognition by hosts, we predicted that cuckoos would lay eggs randomly with respect to nest contents.

METHODS

Study Area and Species

This study was performed in Zhalong National Nature Reserve (46°48′–47°31′N, 123°51′–124°37′E) located on the northern Songnen Plain in Heilongjiang Province, northeast China during the breeding season (May to August) 2014. The habitats include reed swamps, open water and grasslands with a mean annual precipitation and temperature of 426 mm and 3.2 °C, respectively (Yang, Huang et al., 2016). The study subjects were the common cuckoo and its host the Oriental reed warbler. The common cuckoo is the only parasitic cuckoo in this area while the Oriental reed warbler is its predominant host, with a parasitism rate ranging from 34.3% to 65.5% (Liang, Yang, Wang, & Liang, 2014; Yang, Li et al., 2014). Furthermore, in this system the cuckoo eggs strongly resemble the host eggs (Li et al., 2016; Yang, Li et al., 2014).

Model Manufacture

All the models we used for the experiment below were made by the same material (i.e. polymer clay) with the same mass. Three types of models were made: egg shaped, stick shaped (cylindrical) and coin shaped. The egg-shaped models were either blue or white and the same size as the host eggs. They were used in both the parasitism experiment and the experiment for attracting cuckoo parasitism (see below). The stick-shaped or coin-shaped models were white and were only used in the experiment for attracting cuckoo parasitism. We chose blue or white because these colours are nonmimetic to the host eggs, and our previous studies found that blue and white colours are common egg phenotypes for cuckoos in Asia (Yang, Huang et al., 2016; Yang et al., 2010; Yang, Su et al., 2015; Yang, Wang, Cheng et al., 2015). We chose stick or coin shapes as models because they have been used as foreign objects in previous experiments (Yang, Wang, Liang, & Møller, 2015). For more details about the manufacture of stick- and coin-shaped models, see Yang, Wang, Liang et al. (2015).

Parasitism Experiment

The Oriental reed warbler was formerly classified as a subspecies of the great reed warbler, *Acrocephalus arundinaceus* (Dyrcz & Nagata, 2002). Its rejection rate of cuckoo eggs varies from 60% to 100% (Li et al., 2016; Lotem et al., 1992, 1995), which is similar to that of the great reed warbler, which also varies from 60% (Karcza, Moskát, Cherry, & Kisbenedek, 2003) to 100% (Moskát, Szentpéteri, & Barta, 2002). We performed the parasitism experiment in our study population. Three experimental groups were used: (1) the blue model egg group which contained one blue model egg inserted into the host nest replacing one host egg; (2) a white model group with one white model egg inserted into the host nest replacing one host egg; and (3) a control group of host nests that was visited in similar manner without artificial parasitism. The experiment was performed on the day after completion of the host clutch, and the result was classified as rejection if the model egg was ejected, pecked or left cold in the nest (i.e. deserted), or acceptance if the model egg was still being incubated after a 6-day monitoring period.

Experiment for Attracting Cuckoo Parasitism

Nests of Oriental reed warbler were found by systematic searches of reed habitat or monitoring reproductive activities of hosts. Observed nests were monitored to confirm the initiation of egg laying. Two groups were established. For group 1, two host nests that were previously collected as deserted nests were placed near one active nest when its first host egg was found. Two blue egg models and two white egg models were inserted into these two collected nests, respectively. Finally, the combination of the three host nests (one active host nest and two collected nests) were placed in a triangular position 1 m from each other (Fig. 1). We simultaneously set up another combination of nests 10 m away in a random direction with the same nest contents and position, but as all three nests were collected there was no host activity (Fig. 1). For group 2, all procedures were similar to those in group 1, except that the blue and white egg models were replaced by white stick and coin models, respectively (Fig. 1). These treatments were used to ensure that the hosts could easily distinguish their own eggs from model eggs. Therefore, for each group two different kinds of combinations of nests were established, one with and one without an active host. This design allowed us to test the effect of host activities on nest selection during cuckoo parasitism. Furthermore, the nest combination of group 1 consisted of a real host egg, a blue egg model and a white egg model, which allowed us to test the effect of egg appearance on nest selection during cuckoo parasitism. However, in group 2 stick and coin models were used to test the effect of shapes of nest contents on the preference of cuckoo parasitism. All these combinations of nest were monitored for 6 days to confirm parasitism by cuckoos, with a checking frequency of four times per day (i.e. once early morning, once at noon, once in the afternoon and once at dusk). To avoid mutual influence between groups 1 and 2, they were conducted asynchronously (i.e. group 1: mid-June to early July; group 2: mid-July to early August). For collected nests with host eggs in group 2, the eggs were collected from deserted nests.

Statistical Analyses

Generalized linear mixed models (GLMM) and chi-square tests were used to test for a preference of nest selection during cuckoo parasitism and to compare parasitism frequency, respectively. In GLMM different egg appearance/nest contents were nested in the nest combination ID. Then the appearance/nest content and its interaction with parasitism day (i.e. day of cuckoo parasitism during monitor days from 1 to 6) were treated as a fixed effect while nest combination ID and egg-laying date were random effects. For combinations of nests when more than one nest was parasitized, only the frequency of the first parasitized nest was taken into account. Furthermore, for combinations of nests with more than one case of parasitism, the data were analysed similarly by GLMM with