Individual female common cuckoos *Cuculus canorus* lay constant egg types but egg appearance cannot be used to assign eggs to females

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Females of the obligate brood parasitic common cuckoo *Cuculus canorus* are assumed to lay eggs of consistent colour and pattern and egg characteristics have been used to separate between different individuals. We tested the "constant egg-type hypothesis" in blind tests using test persons who grouped cuckoo eggs into "potential clutches" based on similarity in appearance. A correct classification of eggs laid by known (radiotagged) females supported the hypothesis. However, comparisons between maternity based on visual assessments and DNA-based parentage analyses revealed rather poor concordance between the two methods. Our findings indicate that egg characteristics cannot be used to separate between cuckoo females, even if they lay eggs with constant appearance. The reason is probably that there are only small or negligible variations in egg appearance between some females like mothers and daughters or other closely related individuals.

The common cuckoo *Cuculus canorus* (hereafter cuckoo) is an obligate brood parasite, laying one egg in each nest during the host's egg-laying period (Chance 1940, Wyllie 1975, 1981). It has been hypothesized that the long history of interactions between the cuckoo and its hosts has led to a co-evolutionary arms race (Dawkins and Krebs 1979, Davies and Brooke 1989a,b, Rothstein 1990). Even though the cuckoo is known to parasitize more than 100 host species (Wyllie 1981, Cramp 1985, 1992, Alvarez 1994, Moksnes and Røskaft 1995), relatively few species are regularly parasitized in Europe (Moksnes and Røskaft 1995). Individual cuckoo females are thought to interact with only one or a few host species mimicking their eggs (Baker 1942, Wyllie 1981, Brooke and Davies 1988, Moksnes and Røskaft 1995). Based upon appearance of egg-morphs, the cuckoo has been divided into at least 17 different distinct gentes (singular: gens) in Europe (Wyllie 1981, Alvarez 1994, Moksnes and Røskaft 1995, Antonov et al. 2007). Genetic support for the existence of female host-specific races has been obtained by Gibbs et al. (2000).

Based on field observations, it has been assumed since a long time that individual cuckoo females would lay eggs with a relatively constant appearance (Latter 1902, Chance 1940, Baker 1942, Lack 1968, Wyllie 1981), which has resulted in the "constant egg-type hypothesis". Egg appearance has therefore been used as a cue to distinguish between eggs of different cuckoo females (Chance 1940, Molnár 1944, Wyllie 1981). The similarity of eggs has also been used to calculate clutch size (Baker 1942, Wyllie 1981). However, the hypothesis of constant egg appearance at the individual level has never been critically tested. In the present study, we tested the "constant egg-type hypothesis" by using test persons who visually assessed and classified cuckoo eggs that had been laid by known females. Furthermore, we tested if this method can be used to separate between eggs from different females by comparing maternity estimates based on visual assessments of egg appearance with those obtained by using microsatellite DNA markers (see Skjelseth et al. 2004).

Methods

The fieldwork was carried out in an area surrounding artificial fish ponds near the village of Luzice (47°40′N, 16°48′E) in southern Moravia, Czech Republic, from the beginning of May until the end of July in the period 1995–98. This area provides nesting sites for dense populations of four sympatric species of *Acrocephalus* warblers, the great reed warbler *Acrocephalus arundinaceus*, reed warbler *A. scirpaceus*, marsh warbler *A. palustris* and sedge warbler *A. schoenobaenus*, which are all commonly parasitized by the cuckoo (Kleven et al. 2004). For a closer description of the study area and general methods, see Øien et al. (1998).

During the 1995 season, photographs were taken of 38 cuckoo eggs from 38 host nests with a 50 mm lens under standardized conditions (distance 0.3 m, shadowed). In 1996, 1997 and 1998 the numbers of photographed cuckoo eggs were 53, 56 and 25, respectively (from the same number of host nests). The pictures were taken with a 100 mm lens (in 1:2.5 and 1:3 scale, shadowed) using a Canon ML-3 ring flash. These eggs were photographed together with a Kodak colour separation guide and grey scale card which makes it possible to calibrate colour and light conditions on the photographs. All photographs were taken in the field with the cuckoo egg on a grey plate with a 135 negative colour Kodak gold 100 ASA film and processed together every year in the same laboratory. All lenses were multi-coated against UV light.

From a total of 172 photographed eggs, we knew the identity of the laying cuckoo female in 24 cases. This was possible because seven females were radiotagged and observed during egg laying. Two females in each of the years 1995, 1996 and 1997 and one female in 1998 were trapped and equipped with radio transmitters. The radio transmitters (manufactured by Televilt AB, Sweden) weighed 2.75 g, which comprised approximately 2.5% of the body mass of the female cuckoos, and were attached to the central tail feather of the birds (see Honza et al. (2002) for closer methodological descriptions). Additionally, females were marked with a coloured wing tag for visual identification.

Twentysix egg laying episodes of these seven females were observed by radio-tracking, but two of their 26 eggs were ejected by the hosts before photographs could be taken. The number of photographed eggs from each of these females were: 2 (1 found in a marsh warbler (MW) nest and 1 in a reed warbler (RW) nest), 2 (MW), 6 (MW), 1 (RW), 2 (MW), 7 (6 RW and 1 in sedge warbler (SW)) and 4 (SW) (see also Vogl et al. 2002). Eggs from each female were found in one year only.

When examining the "constant egg-type hypothesis", each cuckoo egg was cut out from the photo and assigned with a random number on its back. This was done for all of the 172 cuckoo eggs which consisted of 34 from the great reed warbler, 96 from reed warbler, 29 from marsh warbler and 13 from sedge warbler nests, including 24 eggs observed to be laid by the radio-tracked females. We used two different approaches to examine the hypothesis. First, three test persons with experience in egg assessment (G. R., A. M. and E. R.) classified the cuckoo eggs into groups consisting of eggs with similar appearance, on the basis of a general impression of ground colour and marking pattern (test I). Even though the test persons were not blind with regard to the tested hypothesis they did not know the laying dates and the female identity of the eggs, therefore we assume that their judgement was unbiased. Each group of eggs with similar appearance was assumed to belong to a particular cuckoo female, and was thereafter called a "potential clutch" of cuckoo eggs. "Potential clutches" that were similar between years were classified as belonging to specific females that were assumed to be present in the study area for more than one year. The assessments were done by the three persons together and in the majority of the cases there was no disagreement about the separation into clutches because most egg-types were distinct and easy to classify into different groups. However, for 12 eggs (6.9%) there was disagreement between the three test persons. Since these eggs were assigned to the same group by two of the test persons a "majority decision" was adopted.

First, to test whether the eggs classified into a "potential clutch" may have originated from an individual female, we checked the respective laying dates and whether the eggs had been observed to be laid by radiotagged females. Cuckoo females lay an egg every other day (Wyllie 1981). If cuckoo females have constant egg-types and the assessment of "potential clutches" is reliable, eggs assigned to a clutch should not stem from layings on two consecutive days. Furthermore, all eggs observed to be laid by one radio-tagged female should have been assigned to the same clutch. Given the complexity of the approach, no exact probability estimates of random assignment of eggs into individual clutches could be calculated.

Second, one experienced test person (B.G.S), who did not have any prior knowledge of the egg material was asked to group all 24 eggs laid by the seven radiotracked females (test II). For statistical reasons information was forwarded that there were seven females included in total and that these females had laid seven, six, four, two, two, two and one eggs, respectively. The probability of assigning all the eggs into the correct groups by chance when having this information is given by $_nP_{n1,n2,n3,n4,n5,n6,n7} = n/\Pi n_i!$ (Zar 1999), which yields $_{24}P_{7,6,4,2,2,2,1} = 24/7!6!4!2!2!2!1!$.

Blood samples were obtained from adult cuckoos caught in mist-nets and nestlings via alar venipuncture. We were able to obtain blood samples of seven cuckoo chicks, which had hatched from eggs laid by (one case) or classified as being laid by (six cases) the radiotracked females on the basis of assessments of egg appearance. However, these six eggs were not among those which were directly observed being laid by these females, but they were assessed to belong to "potential clutches" laid by them. In all these six cases the maternity was determined by DNA-analyses. This sample could thus be used to compare maternity estimates determined by genetic analyses with those determined from visual assessments of eggs (for one chick, mentioned above, where the maternity was determined by DNA-analysis, we lack photo of the egg from which it hatched). The main point here is that eggs assessed to belong to the same clutch should have been laid by the same female. The methods for DNA- analyses and all the results from this study have been described in detail by Skjelseth et al. (2004). Six microsatellite loci with cuckoo specific primers Ccμ 02, Ccμ 60, Ccμ 100, Ccμ 108, Ccμ 119 and Ccμ 137 were used (for further description of the DNA analyses see Gibbs et al.

1998). The program Parentage (Chakraborty et al. 1988) was used to conduct the parent exclusion analyses (see also Alderson et al. 1999).

Results

In blind test I, the test persons assessed the 172 cuckoo eggs as belonging to 17 "potential clutches". Eggs from two "potential clutches" were found in all four years, eggs from two others in three years, from seven in two years and eggs of six "potential clutches" were found in one year only. Two "potential clutches" within a year were larger than 10 eggs (17 and 13 eggs, respectively).

Eggs in clutches assessed by the test persons were laid on every second day or at longer time intervals, as expected for the cuckoo, with one exception; two eggs assessed to belong to the same "potential clutch", had been laid on the same day. This indicates that different females had laid these two eggs and that this assessment was wrong. All 24 photographs of eggs that had been observed to be laid by the seven radiotagged females were correctly assessed as belonging to seven different clutches. Hence, eggs in each of these clutches were similar to each other and the difference between the clutches was sufficient for separating them by the human eye. Egglaying by the radiotagged females was observed over an interval of 2, 10, 14, 16, 24, and 28 days, respectively.

In test II, where only eggs laid by the seven radiotracked females were included, all 24 eggs of the seven females were assigned to the correct groups and the probability for a correct assignment by chance is $P = 3.44 \times 10^{-8}$.

Three of the eggs from which the maternity of the hatched chick could be determin by DNA analyses, were assessed to belong to the same "potential clutch". They could thus be used for testing the "constant egg-type hypothesis" stating that they had been laid by the same female. However, the DNA analyses showed that these three eggs had been laid by two females. In addition, one of these females (the one with two eggs) also turned out to be the mother of a nestling hatched from a fourth egg assessed to belong to another "potential clutch". Finally, the DNA-analyses showed that the two remaining eggs were laid by another two different females and they were classified by assessments to belong to another two "potential clutches". As stated by Skjelseth et al. (2004), the statistical power in these analyses was sufficient to confidently assign parentage.

Discussion

The concordance between classifications of egg photographs into "potential clutches" in tests I and II and the known clutches of radio-tracked females suggests that, in this sample, egg appearance was similar within each female and different between females. Furthermore, cuckoo females laid eggs of similar appearance during the entire breeding season, or at least in the course of 28 days even if they laid in the nests of different host species. These findings strongly support the "constant egg-type hypothesis". However, when including the eggs from unknown cuckoo females, two eggs assessed to belong to the same "potential clutch" must have originated from two different females because they were laid on the same day.

The results from the DNA analysis showed less support for the "constant egg-type hypothesis", mainly because one female (classified by DNA) had laid eggs that were assessed (visually) to belong to two different clutches (two eggs in one "potential clutch" and one egg in another). However, the eggs of the three other females were assessed to belong to different "potential clutches". Since our sample was small, it would not be justified to refute the "constant egg-type hypothesis" on this basis. The parentage analyses based on DNA is merely a test of our method of egg assessment by humans and this finding indicates that visual assessment of eggs is not sufficiently accurate to separate reliably between different females. One reason might be that closely related females such as mother and daughter or sisters may produce similar eggs (Jensen 1966, Gosler et al. 2000). This may cause only minor differences in egg type between some cuckoo females, which raises our error probability when assigning eggs to potential clutches by visual cues. Even when using more objective measures for egg characteristics than in this study, it might be difficult to separate between females. For instance, Cariello et al. (2004) were able to assign only 55% of the studied eggs of guira cuckoos *Guira guira* to the correct mother using egg dimensions and eggshell pattern. It is also worth mentioning that the blind test II, yielded clear support for the "constant egg-type hypothesis", and that the DNA analyses were based on different samples, which mean that the results are not directly comparable.

Constant egg types have so far been documented only in a few bird species. In the village weaver *Ploceus cucullatus*, Collias (1993) showed that females did not change their overall egg appearance throughout lifespan and Jackson (1992) found constant egg types in individual females of the northern masked weaver *Ploceus taeniopterus*. In both these species, intraspecific brood parasitism (egg dumping) is common.

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