

## Egg characteristics are unreliable in determining maternity in communal clutches of guira cuckoos *Guira guira*

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Egg characteristics have been used to determine egg maternity for birds in situations where two or more females lay eggs in a single nest (as in communal breeders and intraspecific parasites). We assessed the applicability of egg morphometry and eggshell appearance in ascribing egg ownership in communal clutches of guira cuckoos *Guira guira*, a species where up to seven females may lay eggs in a joint nest. We used both combined variables (including egg mass, length, width, shape and two eggshell variables), and a shape index to test whether eggs laid by each female were similar but different from eggs laid by other females. Also, we conducted discriminant function analyses to verify if eggs could be correctly classified to their mothers based on their characteristics. The correct maternity was determined by yolk protein electrophoresis of freshly-laid eggs. Individual female clutches were separated through egg characteristics or shape alone in 29% and 41% of the groups tested, respectively. Differences were mostly due to a single female that differed from her nest-mates in a unique egg variable. On average, 55% of the eggs analyzed were not assigned to the correct mother using egg dimensions and eggshell speckling pattern. In conclusion, the egg characteristics used do not reliably indicate maternity in guira cuckoo communal clutches. We therefore recommend a protein-based verification of egg appearance characteristics for assigning maternity in other species in which multiple female laying occurs.

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In communal and polygynous breeders with complex reproductive tactics (reviewed in Brown 1987), or in cases of intraspecific nest parasitism (reviewed in Yom-Tov 1980), several females of the same species may lay eggs in a joint nest. In these situations, egg maternity identification is necessary to evaluate reproductive tactics used by different females.

One way to identify individual female clutches in such situations is by egg appearance (e.g. colour, shape, size,

speckling pattern). Egg characteristics may be a good predictor of maternity in species where inter-individual variation is high and intra-individual variation is low. This has been reported, for instance, for the herring gull *Larus argentatus* (Baerends and Hogan-Warburg 1982), the ring-billed gull *Larus delawarensis* (Fetterolf and Blokpoel 1984), the northern masked weaver *Ploceus taeniopterus* (Jackson 1992), and the emperor goose *Chen canagicus* (Petersen 1992).

For some bird species, however, there may be high variability in egg characteristics within clutches laid by the same female, limiting the use of such variables for correctly ascribing maternity. In particular, a female's first egg in the clutch (Henriksen 1995) or most commonly the last egg (Coulson 1963, Meathrel and Ryder 1987, Viñuela 1997) are frequently different from her other eggs. Thus, there is a risk of assigning odd eggs in a clutch to another female, especially if the laying sequence is unknown (Brown and Sherman 1989, McRae 1997). For this reason, in avian species where no evaluation of egg variability within and among female clutches has been done, egg appearance possibly may not be used to discriminate among eggs laid by potentially different females.

Populations of the South American communally-breeding guira cuckoo *Guira guira* in central Brazil reproduce during the rainy season, from mid-August to mid-March, with groups frequently renesting several times (Macedo 1992, Melo 1997). Reproductive guira cuckoo groups of two to 15 related and unrelated individuals use a joint nest and exhibit both cooperative as well as competitive behaviors, such as egg ejection and infanticide (Macedo 1992, 1994, Melo and Macedo 1997, Macedo et al. 2001). Approximately half of all eggs laid are ejected especially at the onset of laying. Joint nests may contain up to 26 eggs; average egg weight is 25.3 g (approximately 16% of adult weight), and average egg length and width are 42.5 mm and 31.8 mm, respectively (Macedo 1992). The eggs are turquoise, overlaid with a lace-like calcareous pattern of white splotches and streaks of considerable thickness (speckling), varying widely among eggs. During egg laying it is common to find two, three or even four eggs laid in a joint nest on the same day. The identification of clutches from individual females in communal nests of guira cuckoos is important to understand their reproductive strategies and social organisation.

Recently, a new methodology for determining maternity of guira cuckoo eggs through egg yolk protein electrophoresis was developed (Cariello et al. 2002). This technique uses yolk proteins in fresh, non-incubated eggs, allowing sampling of eggs before ejection. Additionally, capturing females is not necessary (egg proteins are of maternal origin only), which is particularly useful in the case of guira cuckoos, where capturing adults is very difficult and favours males, thus reducing the possibility of sampling female tissue for DNA analyses.

Our objective in this study is to evaluate the applicability of egg characteristics for ascribing egg ownership in communal clutches of guira cuckoos. The maternity of eggs was determined by electrophoresis of yolk proteins.

## Materials and methods

### Study area and field methods

The 30 km<sup>2</sup> study area is located near Brasília, Distrito Federal, Brazil (15°47'S, 47°56'W; altitude 1,158 m), and is characterized by a mosaic of native "cerrado" vegetation, urbanized plots and cultivated fields. In this area, peak reproductive activity of guira cuckoos occurs in September and October (Macedo 1992). Details of field methods are described elsewhere (Cariello et al. 2002). Briefly, in both field seasons, 1998 and 1999, we placed fishing nets below nests for collection of ejected eggs. From August to December, fresh non-ejected eggs were collected from nests and replaced with dummy eggs, and fresh ejected eggs were collected from the nets without substitution. The introduction of dummy eggs did not disrupt patterns of egg laying and ejection (details in Macedo et al. in press). Collection of eggs was authorized by IBAMA/Brazil (076/98-DIFAS and 027/99-DIFAS permits).

### Egg characteristics

Fresh eggs collected in the field were taken to the laboratory where we measured maximum length (L) and width (W) with a digital caliper (precision 0.1 mm), and weight with a digital scale (precision 0.1 g). A shape index was calculated using the ratio of maximum diameter to length multiplied by 100 (Romanoff and Romanoff 1949), and logarithmically transformed in all analyses (Zar 1999).

We photographed eggs using a 35 mm Canon camera with 50 mm lens with two (+1 and +2) added magnification rings. All photographs were taken against a neutral background and at minimum focal distance. We developed the photographs on paper, scanned them, and analyzed images with the software UTHSCSA Image Tool for Windows (version 2.00 alpha 3). The scanned photographs of eggs had their maximum length and width calibrated appropriately by the software, and two speckling pattern measures were taken of each egg image by one person only. First, the average percentage of white speckling ( $n = 5$  measures) in a square at the midsection of the egg was measured. The square root of each average percentage was arcsine transformed for all analyses (Zar 1999). Second, we counted the number of times colour changes (blue/white) on the eggshell occurred along a transect perpendicular to the long axis of the egg at 1/2 of egg maximum length. The length of the transect was equal to the total egg width at that point. Since most guira cuckoo eggs are equally elliptical at both ends, any measure of speckling pattern originating at the upper or lower quarters of the egg, or at the blunt or pointed ends (e.g., diameter at 1/4 or 3/4 length from a specific end) is ambiguous in our study. Thus,

although it would have been desirable to have a higher number of speckling variables measured, the guira cuckoo eggs with symmetrical ends prevent using them as points of reference.

After photographs were taken, we broke the eggs, weighed the yolk and albumen separately and froze them at  $-20^{\circ}\text{C}$ . Eggshells were weighed and stored at room temperature. This procedure was required for objectives other than those in this study. Yolk samples were further used in electrophoresis analysis of proteins for egg maternity identification (see below). However, for maternal identification only, minute yolk samples may be obtained by puncturing the eggshell with a needle without embryonic damage (see Schwabl 1993 for details).

### Female identification

The yolk protein electrophoresis analyses for maternal identification of guira cuckoo eggs are detailed in Cariello et al. (2002). Succinctly, yolk samples from recently laid guira cuckoo eggs were digested by trypsin, incubated, and heated to  $94^{\circ}\text{C}$  for 30 seconds to stop digestion. Samples diluted with loading buffer were heated at  $94^{\circ}\text{C}$  for 90 seconds, cooled on ice, and then run on 12% denaturing polyacrilamide gels (Laemmli 1970) for 2 h at 20 mA and 80 V at room temperature. Protein fragments were visualized by silver staining (Morrissey 1981). Yolk proteins in freshly-laid eggs are maternally derived, and thus identical patterns of bands indicated eggs laid by the same female, while different patterns indicated eggs laid by different females.

### Statistical analyses

In statistical analyses we used all six egg characteristics measured: mass, length, width, shape, white speckling (%), and number of colour changes. Discriminant function analysis was used to determine whether egg characteristics can distinguish eggs laid by each female within a group, and how well these variables discriminate individual clutches. This procedure yields jackknifed classification matrices to determine the percentage of correct assignment to mothers in each group (Tabachnick and Fidell 1996). Means of all egg characteristics were compared among females of the same breeding group by multivariate analysis of variance (MANOVA). Additionally, we conducted a univariate analysis of variance (ANOVA) for testing whether shape alone differed for eggs laid by separate females. We treated the variable shape separately because it is often one of the characteristic most used by researchers to discriminate among eggs of different females (reviewed in Yom-Tov 1980). All statistical analyses were conducted with SYSTAT software (version 9.0).

## Results

We collected 297 eggs from 48 nests of 24 guira cuckoo groups during 1998 and 1999. As mentioned above, the breeding season for guira cuckoos in our study area ranges from mid-August to mid-March, and our study ended in mid-December, allowing the population to continue breeding throughout the rest of the season. Thus, there is little likelihood that the study adversely affected the local population. Overall mean values ( $\pm\text{SD}$ ;  $n$ ) for each variable based on all eggs from all clutches were: mass 24.3 g ( $\pm 2.9$ ;  $n = 221$ ), length 42.7 mm ( $\pm 2.1$ ;  $n = 242$ ), width 31.8 mm ( $\pm 1.3$ ;  $n = 241$ ), shape index 74.7 ( $\pm 3.6$ ;  $n = 241$ ), percentage of white speckling in the mid-section of the eggshell 0.54 ( $\pm 0.11$ ;  $n = 185$ ), and number of times colour change occurs along a transect at  $1/2$  of the egg length 1.067 times per mm ( $\pm 0.267$ ;  $n = 185$ ).

Maternity was identified for a total of 195 fresh-laid eggs in 34 nests from 22 groups. However, for the ANOVA, MANOVA and discriminant functions, we did not include nests where only a single female laid eggs, or nests where all females each laid a single egg because, in the first case, we had no other female for comparative purposes, and in the second case, there was no variance available for the analyses. Thus, for analyses we used 177 eggs belonging to 28 communal clutches from 17 guira cuckoo groups. Eight groups renested in 19 breeding attempts during our study period, and for those we considered all their repeated nesting bouts within a year together in the analyses. In these cases, our intention was also to verify whether the same female could be identified as a layer in subsequent renestings of her group based on appearance of her eggs. This identification would be important since not all guira cuckoo females participate in all nesting bouts of their groups and some females lay more eggs in communal clutches than others (Cariello et al. 2002, Macedo et al. in press). As a consequence of pooling nesting attempts, the power of analyses increased because a higher number of eggs per female was analyzed (Zar 1999). To confirm that pooling nesting attempts increased the power of analysis and to test whether pooling eggs across clutches did not increase within-female variation, we also analysed the data by considering nests individually. We found that percentage of nests where at least one female differed significantly from her nest-mates with respect to egg appearance was smaller when nests were analyzed separately (17% for discriminant analyses and 33% for ANOVA for shape) than when nesting attempts were pooled (see results below). Thus, for the purposes of this paper (i.e. to test whether there is less variability within eggs of a single female versus among eggs of different females), we used the results from the pooled nests of each group.

Table 1. Reproductive parameters of the 17 guira cuckoo groups monitored in 1998 and 1999 in central Brazil and used in the uni- and multivariate analyses of variance, and in the discriminant functions for determining maternity of eggs based on egg characteristics. The correct maternity was determined by yolk electrophoresis (see Materials and methods).

Year	Group	Number of nesting bouts	Total eggs in communal clutches	Number of laying females
1998	1	1	9	4
	2	1	4	2
	3	1	5	2
	4	3	28	6
	5	1	14	5
	6	3	25	10
	7	1	11	5
	8	1	15	5
	9	1	15	6
	10	2	3	2
1999	11	3	22	6
	12	2	24	5
	13	2	9	3
	14	1	4	3
	15	2	15	4
	16	2	17	5
	17	1	6	2

The number of laying females in the groups studied averaged 4.4, ranging from two to 10. Breeding groups laid on average 13.3 eggs per season, ranging from three eggs laid by Group 10 over two nesting bouts to 28 eggs laid by Group 4 distributed over three bouts (Table 1). In general, discriminant function analyses yielded low percentages of correct classifications of eggs to their mothers (22 to 75%), averaging 44% (Table 2). In only

29% of the groups were individual female clutches within communal nests separated through egg characteristics by the MANOVA analysis (Table 2). In these cases, however, the most useful variable for discriminating among females differed among the groups (mass, length, width or colour changes per mm) and, in most cases (83%), the significant result was due to a single female that differed from others in her group. For instance, in Group 6, where at least 10 guira cuckoo females contributed 25 eggs to the communal clutches in a season (from which 24 were analyzed), only female E laid eggs that differed from those laid by females A, C, G and H. Eggs laid by female E had shorter length, and no other females differed in this group for this or other egg variables. In Group 9, female B laid broader eggs compared to eggs laid by females A, D and F, and this was the only difference among eggs laid by the six females in this group (Table 2). For those groups where MANOVA separated at least one laying female from the others based on egg appearance, there was, on average, 6.4 females, a significantly higher number compared to the average 3.6 laying females in groups where no difference between females was detected in any egg variable ( $t$ -test,  $t = -2.77$ ,  $P = 0.034$ ,  $n = 17$ ). This indicates that with more females in a group, there is a greater chance that eggs from one or two consistently differ from those of nest-mates with respect to at least one egg variable.

The egg shape variable was distinct among females in 41% of the analyzed groups. The only sources of differences detected included cases where two females differed from each other (43% of the cases) or a single

Table 2. Percentage of correct maternal classification of eggs, and difference among individual female clutches based on egg characteristics in 17 communally-breeding guira cuckoo groups<sup>1</sup>.

Year	Group	Correct classification of all eggs (%) <sup>2</sup>	Existence of difference among females within groups <sup>3</sup>	Most important variable discriminating females	Different females <sup>4</sup>
1998	1	22	no	—	—
	2	75	no	—	—
	3	40	no	—	—
	4	52	yes	mass/color changes per mm	B ≠ D; C ≠ D/A ≠ B; B ≠ C; B ≠ D
	5	62	yes	mass	A ≠ B; A ≠ E; B ≠ C; B ≠ D; C ≠ E; D ≠ E
	6	38	yes	length	A ≠ E; C ≠ E; E ≠ G; E ≠ H
	7	33	no	—	—
	8	23	no	—	—
	9	64	yes	width	A ≠ B; B ≠ D; B ≠ F
	10	67	no	—	—
1999	11	50	no	—	—
	12	53	yes	length	A ≠ B; A ≠ C; A ≠ D
	13	44	no	—	—
	14	25	no	—	—
	15	33	no	—	—
	16	36	no	—	—
	17	40	no	—	—

<sup>1</sup>See Table 1 for number of eggs and females.

<sup>2</sup>By jackknifed classification matrix.

<sup>3</sup>MANOVA using Wilks'  $\lambda$  test; significant level at  $P < 0.05$ .

<sup>4</sup>Each letter designates a single female within her group.

female differed from the others in her group (57% of the cases; Table 3). In Group 12, only female A laid eggs with different shape compared to those laid by females B, C, D and E. Her eggs were broader and shorter compared to the eggs laid by other females in this group.

Thus, eggs laid by the same guira cuckoo female may be highly different with regard to some traits (Fig. 1A), while eggs laid by different females within a group may be quite similar (Fig. 1B). This makes it impossible to use egg characteristics to reliably determine individual clutches in most communal nests of this species, either through simple visual inspection of the eggs or by quantitative statistical analyses.

## Discussion

With the use of allozymes (Manwell and Baker 1975, Fleischer 1985, Fleischer and Smith 1992) or DNA analyses (McRae and Burke 1996) to identify females, it has been found that there is lower variation in egg morphometrics within a female's clutch than between clutches of different females in several species. For guira cuckoos, however, we found that egg maternity, determined through yolk protein electrophoresis, could not be correctly and/or consistently assigned using egg characteristics. On average, less than half of the eggs within breeding groups were assigned correctly to the mother. Additionally, the few differences detected among reproductive females within groups were due mostly to a single female differing from one or more of the other laying females with regard to one egg variable, and such differences only occurred in groups where a higher number of females contributed eggs to the communal

clutch. Thus, our results indicate that egg characteristics are not a reliable criterion to discriminate individual female clutches in guira cuckoo communal nests and should not be used for this purpose in this species.

In two other crotophagine species, however, egg appearance has been used to distinguish the eggs of different females. In communal nests of groove-billed anis *Crotophaga sulcirostris*, Vehrencamp et al. (1986) felt confident in their assignment of maternity based on egg characteristics because groups are usually stable, most of their birds were marked and sexed, and they found a consistent relationship between the number of females in the group, the total number of eggs laid in a given nest, and the number of egg types (Vehrencamp 1976). Loflin (1983), studying the communally breeding smooth-billed ani *Crotophaga ani*, also identified individual females based on egg dimensions, colour, shape and weight, in addition to total communal clutch size. It would be interesting to assess egg variability for females in the anis using modern molecular techniques, as maternal identification of eggs provides important data with implications for reproductive skew theory. For guira cuckoos, as proposed previously for the two other crotophagine species, communal clutch size and number of laying females are positively correlated (Cariello et al. 2002). However, the high variability in features of eggs laid by the same female indicates that this is an inaccurate method for ascribing maternity in this species.

After assurance that variation in egg measurements among females is higher than variation within females, researchers could safely use egg characteristics for detecting intraspecific nest parasitism in some bird species (Baerends and Hogan-Warburg 1982, Fetterolf and Blokpoel 1984, Jackson 1992, Petersen 1992). For

Table 3. Comparison of shape variables among eggs laid by different females in communal clutches of 17 guira cuckoo groups monitored in 1998 and 1999 in central Brazil<sup>1</sup>.

Year	Group	ANOVA test		Different females <sup>2</sup>
		F-ratio	P	
1998	1	2.738	0.176	—
	2	4.685	0.163	—
	3	2.967	0.183	—
	4	1.486	0.252	—
	5	2.044	0.181	—
	6	4.516	0.006*	A ≠ F; A ≠ G; A ≠ I
	7	1.185	0.437	—
	8	3.962	0.046*	trend for A ≠ C
	9	5.561	0.041*	A ≠ B
	10	3.022	0.332	—
1999	11	4.143	0.045*	B ≠ E
	12	10.999	< 0.001*	A ≠ B; A ≠ C; A ≠ D; A ≠ E
	13	10.690	0.011*	A ≠ B; B ≠ C
	14	0.878	0.602	—
	15	4.189	0.079	—
	16	6.374	0.024*	A ≠ C; C ≠ E
	17	0.681	0.470	—

<sup>1</sup>See Table 1 for number of eggs and females.

<sup>2</sup>Each letter designates a single female within her group.

\*P significance at 0.05.



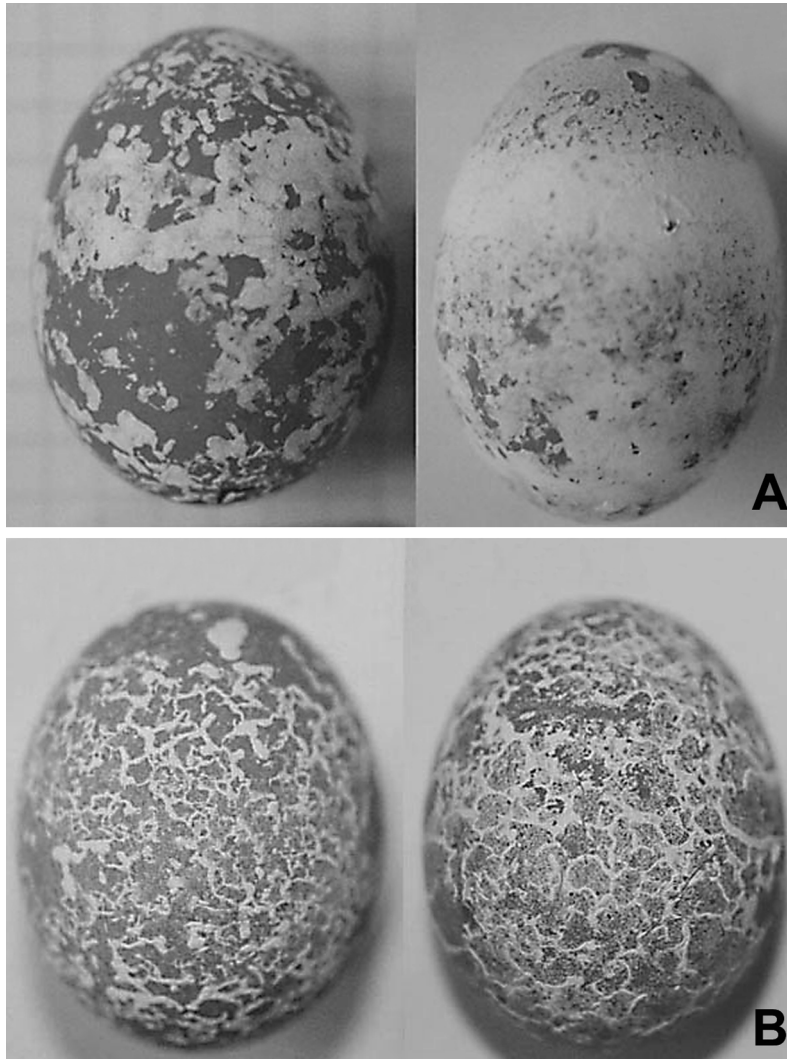


Fig. 1. (A) Two eggs laid by female C in the same nesting bout of Group 5. Egg characteristics for the egg on the left were: mass 21.8 g, length 40.5 mm, width 30.8 mm, shape 76.0, white speckling percentage 0.54, and colour changes/mm 0.748. Egg characteristics for the egg on the right were: mass 22.9 g, length 42.5 mm, width 31.4 mm, shape 73.9, white speckling percentage 0.95, and colour changes/mm 0.389. (B) Two eggs laid by different females in consecutive nesting bouts of Group 13. Egg on the left was laid by female A and its measures were: mass 21.8 g, length 39.0 mm, width 31.8 mm, shape 81.5, white speckling percentage 0.60, and colour changes/mm 1.521. Egg on the right was laid by female C and its measures were: mass 22.8 g, length 40.1 mm, width 31.9 mm, shape 79.6, white speckling percentage 0.69, and colour changes/mm 1.299. In this group, eggs laid by females A and C were not different with regard to their characteristics.

other species, however, researchers have found no evidence that eggs within a single or repeated female clutch are sufficiently similar in appearance to accurately infer maternity (Coulson 1963, Brown and Sherman 1989). Thus, we suggest that egg characteristics be used as a reliable method for ascribing maternity after validation, for instance, by identification of marked females during egg-laying or by biochemical methods such as protein electrophoresis (Fleischer 1985, Fleischer and Smith 1992), or DNA fingerprinting (McRae and Burke 1996).

One of the most important reasons for conducting a previous evaluation of variability in eggs is that in many species one egg in a female's clutch (usually the last one) may differ from the others (Meathrel and Ryder 1987, Viñuela 1997), and such a difference may be greater than that between different females' eggs. In studies of

intraspecific nest parasitism, Brown and Sherman (1989) and McRae (1997) urged caution in assigning the last-laid egg of a clutch to another female based solely on its characteristics, and recommended that egg appearance variables should be used in combination with laying sequence data and daily checking. This methodology may be useful in some intraspecific nest parasitism studies, but constitute an impossible task when identifying individual female clutches in joint nests. In guira cuckoo communal nests, daily checking and yolk electrophoretic analyses allowed us to verify that females: (1) lay eggs at irregular intervals, (2) do not contribute the same numbers of eggs to the joint nest, and (3) do not follow similar patterns of laying. Thus, in this communal nester, there are probably both physiological and behavioural constraints limiting the number of eggs females lay and their order of entering the laying

sequence. This makes it impossible to use laying sequence data to predict the exact number of laying females at a nest in guira cuckoos.

In the context of female reproductive strategies, it is constructive to speculate why guira cuckoo females lay eggs with such high variability. In some species it is thought that the high variation in egg traits among individuals may have evolved as a counteradaptation to nest parasitism. This would function in species with both intra- as well as inter-specific parasitism because females may be able to reject eggs according to their degree of difference in appearance from their own eggs (Rothstein 1990, Møller and Petrie 1991, Lahti and Lahti 2002). In the village weaverbirds *Ploceus cucullatus*, for instance, selection pressures due to cuckoo parasitism may have led to uniform weaver eggs within clutches and greater disparity between clutches (Lahti and Lahti 2002). Egg characteristics of guira cuckoos may be under similar selection pressures but in the opposite direction. It may be profitable, from the individual perspective, to maintain high egg variability within the clutch, thus scrambling egg identity, which then decreases the possibility of egg recognition and possible ejection by nest-mates. Thus, the maintenance of communal breeding in this species may partly derive from individual variability in egg traits. If females cannot recognize their own eggs, then there may be a cost associated with the ejection of any egg from the nest. In other words, in guira cuckoo nests, where up to seven females may lay eggs, if each female had a unique egg pattern and was able to recognize it, possibly they would be able to eject all other females' eggs and communal clutches would fail.

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