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# Individual female clutch identification through yolk protein electrophoresis in the communally breeding guira cuckoo (*Guira guira*)

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**Abstract**

**Avian communal breeding systems generate alternative behavioural strategies for females, resulting in differences in reproductive success. Identifying eggs of different females in such systems is problematic, however, due to egg destruction before incubation, difficulty of capturing adults, and/or inaccuracy of egg identification based on egg morphometry. Here, we describe a technique that uses electrophoresis of yolk proteins to determine egg ownership, which we applied to communally breeding guira cuckoos (*Guira guira*). Validation of the method included identical yolk protein banding patterns in all eggs of the same female, but different patterns in eggs of different females in budgerigars (*Melopsittacus undulatus*), and identical patterns in yolk follicles of the same females in guira cuckoos. We applied the protocol to 195 guira cuckoo eggs from 34 joint nests in 2 years. All multiple guira cuckoo eggs laid on the same day in single nests had distinct banding patterns of yolk proteins, practically eliminating the possibility of more than one female being represented by the same pattern. Some identical banding patterns were repeated in different days within a nesting bout, indicating that some females laid several eggs in shared nests. Identical patterns occasionally occurred in renestings of groups, indicating that some females lay eggs in consecutive nestings. Yolk protein electrophoresis is a useful tool to identify egg maternity in other circumstances, such as polygynous mating systems with joint nests and intraspecific parasitism. Additionally, it is an alternative method for species where electrophoresis of egg white proteins does not show sufficient polymorphism.**

*Keywords*: communal breeding, egg ejection, egg maternity, guira cuckoo, *Guira guira*, yolk protein electrophoresis

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## Introduction

In avian nests where two or more females deposit eggs, it is crucial to identify the ownership of individual eggs to understand the strategies of the laying females and their subsequent reproductive benefits. These situations occur in species with complex social systems, such as communal breeders (Craig 1979; Koenig 1981; Vehrencamp *et al*. 1986; Brown 1987; Brown & Brown 1990; Macedo 1992), or in cases of intraspecific nest parasitism (Andersson & Eriksson 1982; Brown 1984; Møller 1987). In communal reproduction three or more individuals in a group breed simultaneously, sometimes using a joint nest (see Brown 1987 for alternative terminology). In this system, helpers at the nest may include adults related genetically to the nestlings (former offspring that remained in the group), as well as adults unrelated to the brood. In intraspecific nest parasitism females lay eggs in nests of conspecifics without caring for their offspring. The host females are faced with

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the difficulty of discriminating between their own eggs and parasitic ones, which may be very similar in appearance to their own, leading to acceptance of parasitic eggs in some cases and rejection in others (Yom-Tov 1980; Lahti & Lahti 2002).

In some intraspecific nest parasitism studies, egg maternity has been determined through electrophoresis using embryonic tissue (Gowaty & Karlin 1984; Wrege & Emlen 1987; Brown & Brown 1988; McKitrick 1990). However, embryonic sampling may underestimate the contribution of each female to the nest under two conditions: first, when clutch reduction occurs before incubation starts (i.e. before embryo development). For instance, a significant sampling of embryos may not be possible in cases where egg loss due to predation or egg ejection is high at the onset of laying, or in the case of infertile eggs. Second, when the capturing of adults in the study species is unsuccessful or insufficient, as embryonic tissue analysis requires parental sampling for comparison of the results.

In such situations, protein electrophoresis of recently laid eggs can be useful as an alternative method for determining the maternity of individual clutches (Smyth *et al*. 1993; Andersson & Åhlund 2001). Egg sampling in the early stages of laying avoids the loss of information due to clutch reduction. In addition, female capturing is not necessary because proteins of undeveloped (nonincubated) eggs are exclusively maternally derived, and any polymorphism reflects maternal genotype. Therefore, if different protein phenotypes and consequently genotypes occur among eggs within a single nest, it is very likely that more than a single female laid eggs in that clutch (Manwell & Baker 1975).

Allozyme analysis of egg white has been used in studies of avian intraspecific nest parasitism to differentiate between clutches of hosts and parasites in the yellow-billed cuckoo *Coccyzus americanus* (Fleischer *et al*. 1985), the house sparrow *Passer domesticus* (Kendra *et al*. 1988) and the cliff swallow *Hirundo pyrrhonota* (Smyth *et al*. 1993). These studies used the separation of enzyme products of different alleles at a single locus (allozymes) based on electrical charges. Some alleles yielded detectable polymorphism that allowed the identification of more than one laying female in a single nest. Although egg white proteins have been used more widely for identifying individual clutches in nest parasitism studies, yolk proteins may also be used for this purpose. Fleischer (1985) and Fleischer & Smith (1992) used yolk proteins in allozyme analysis in studies of interspecific parasitism to verify if two or more cowbird females parasitized the same host nests. Sufficient polymorphism of egg white proteins may not exist in all species and, in these cases, it has been suggested that polymorphic electromorphs of yolk proteins could be good markers (Fleischer 1985). However, as allozyme analysis may not show sufficient differences among females because different females may possess the same alleles, they have been used mainly for exclusion of maternity, and not for a precise maternal identification of eggs.

Despite its potential importance for studying individual reproductive success and female strategies in birds, electrophoresis of egg white or yolk proteins has never been used specifically for identifying individual female clutches in joint nests of communal breeders. Because allozyme analysis may present the limitation mentioned above, new methods using total proteins rather than allozymes would be better indicated for such studies. More recently, Andersson & Åhlund (2001) developed a very accurate 'protein fingerprinting' technique, based on egg white sampling and isoelectric focusing electrophoresis (IEF) to determine egg maternity of hosts and parasites in nests of the common goldeneye duck *Bucephala clangula*. Through this technique, total proteins of egg white are separated according to their charge in an electric field applied over a stable pH gradient in the gel, and the samples analysed produced a rich banding pattern of egg white proteins that was very variable among females. Thus, methods using total proteins may allow a better resolution of differences among females (compared to allozyme methods) because they test for differences among all proteins present in the sample.

For the past decade, an ongoing study concerning the reproductive strategies of the South American communally breeding guira cuckoo (*Guira guira*) has been carried out in central Brazil (Macedo 1992, 1994; Quinn *et al*. 1994; Macedo & Bianchi 1997a,b; Melo & Macedo 1997; Macedo *et al*. 2001). These birds occur in reproductive units varying from two to 15 adults (mode = 6) and their egg laying and egg ejection patterns resemble those of other species in the Crotophaginae (Davis 1942; Vehrencamp 1977; Sick 1997). Typically, adults in each group build a single nest, in which reproductive females of the group lay their eggs. The communal clutch may range from one to as many as 26 eggs during a single nesting bout. During the breeding season, up to five successive nesting bouts may occur for groups in the same territory (Melo 1997). The social behaviour of group members includes cooperation (e.g. nest construction, predator defence, territorial defence, nestling feeding) as well as competition (e.g. egg ejection, infanticide). The groups are composed of related and unrelated individuals, and reproductive opportunities are not equivalent for all group members because some individuals may not reproduce in some of the nesting bouts (Quinn *et al*. 1994).

Past studies have focused upon clarifying aspects related to individual reproductive success and interactions leading to egg ejection and infanticide within guira cuckoo breeding groups. However, it is impossible to determine the number of females within each group from field observations because the species is sexually monochromatic and monomorphic (Sick 1997). Additionally, the number of females in a group may not be equivalent to the subgroup

that is actually reproducing in any given nesting bout. Added difficulties in this species are (Macedo 1992): (1) adults are not caught easily in nets and also learn to avoid traps; (2) there exists a strong bias in capture, favouring males; and (3) egg ejection is prevalent at the onset of laying, before incubation starts, thus excluding the possibility of using embryonic tissue.

In this study, a new method was employed to ascribe maternal origin in guira cuckoo communal clutches from electrophoretic banding patterns of yolk proteins. To increase resolution of differences among individuals, protein extracts were digested with a protease analogously to the use of restriction enzymes in DNA-based methods of resolving genetic differences. Our specific objectives were to determine the number of females laying eggs in guira cuckoo communal nests, the pattern of laying within nests, and whether females lay eggs in successive nesting bouts of the group. Such methods should be widely applicable to other avian species, and highly suitable in the resolution of problems in evolutionary studies and related areas.

## Materials and methods

### *Study area and species*

We conducted the field study in the central Brazilian Plateau in a semi-urban area of 30 $km^2$ near Brasilia (15°47′ S, 47°56′ W; altitude 1.158 m) that includes fragments of the native Brazilian savanna (known as 'cerrado'), cultivated fields, housing developments and residential gardens. Preferred nesting sites for guira cuckoos in this area include the introduced monkey puzzle trees, *Araucaria angustifolia*. Reproductive activity is restricted mainly to the rainy season, from mid-August to mid-March, with the peak reproductive activity of the guira cuckoos in our study area occurring in September and October.

### *Egg and female collection*

In July 1998 and 1999 we searched for old nests, commonly re-used by breeding groups, and placed nylon fishing nets (mesh = 15 mm) camouflaged with *Araucaria* branches underneath nests in good conditions to collect ejected eggs. From August to December 1998 and 1999 we visited the nests every other day, and once reproductive activity was detected, we visited them daily. We recorded the maximum number of birds seen around the nest in successive visits. This may be considered an estimate of group size, but may be an underestimate as birds were not banded. We collected ejected eggs from the net and nonejected fresh eggs from the nest, which were substituted by dummy eggs. The birds accepted these dummy eggs readily, which were hand-painted chicken and pigeon eggs, or guira cuckoo eggs collected in another area. The dummy eggs were numbered sequentially as they were substituted to record any subsequent ejection. The fresh eggs were taken to the laboratory, measured in length and width, weighed, photographed and then broken apart to separate the yolk and egg white (which were weighed separately and frozen at –20 °C). The eggshell was also weighed and stored at room temperature. For validation of the yolk protein electrophoresis method for the guira cuckoo we collected three guira cuckoo females in another area towards the end of the breeding season. Their ovaries were frozen immediately at –20 °C. The yolk of individual follicles of these ovaries was used to validate that the yolk protein banding pattern of individual females is identical among their follicles and hence their eggs, and that different females have different patterns.

### *Polyacrylamide gel electrophoresis (PAGE)*

To develop the protocol, we used 36 recently laid eggs from nine individually housed captive budgerigars, *Melopsittacus undulatus*. These analyses included eggs laid in the same and in different clutches of known females. We ran entire clutches on single gels to compare yolk protein polymorphism of eggs. We used this protocol then for the guira cuckoo.

We thawed the frozen yolks and punctured their membranes to extrude contents. Yolks were diluted 1:1 with distilled water and homogenized. Samples of the homogenate were then either: (1) mixed with 67 mM potassium phosphate buffer (pH 7.6) in a 1(homogenate):9 (buffer) proportion; or (2) mixed with 1 mM HCl plus trypsin (for protein digestion; SIGMA:bovine pancreas) and 67 mM potassium phosphate buffer in a 1(homogenate):2(enzyme):18 (buffer) proportion. The samples containing trypsin were incubated for 2 h at room temperature, and the digestions were stopped after incubation by heating to 94 °C for 30 s. All samples (with or without the enzyme) were frozen until further analyses.

In PAGE analyses, all samples were diluted 1:4 with loading buffer (0.0625 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol. 0.05% bromophenol blue), heated at 94 °C for 90 s, and then cooled on ice. Gels were run under denaturing conditions using standard procedures based on Laemmli 1970 protocol, i.e. proteins were separated according to their size. Separating gels consisted of 7.5% or 12.0% acrylamide as detailed by Laemmli (1970). Gels ran for 2 h at 20 mA and 80 V at room temperature in Mini-Protean Electrophoresis Cell (BIO-RAD). To maximize sensitivity and resolution, proteins were visualized by silver-staining (Morrissey 1981). To test for instability of the samples and reproducibility of the protein digestion procedure, replicate digestions and analyses were conducted on freeze/thawed yolk samples.

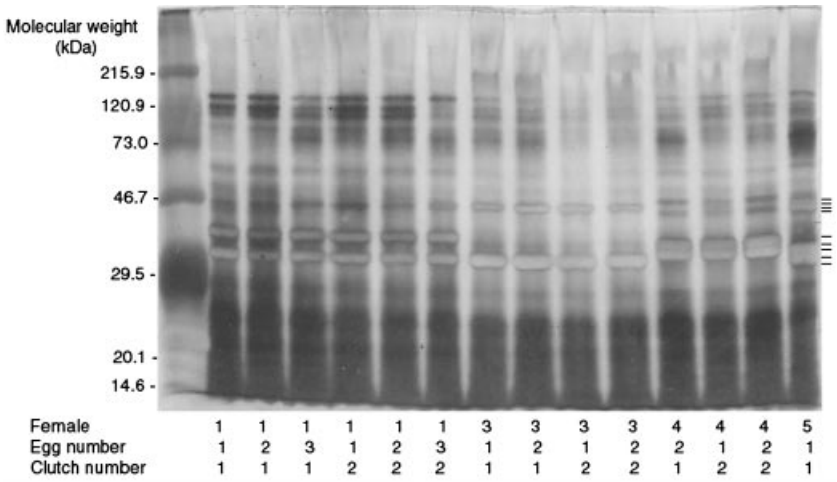


**Fig. 1** SDS-PAGE of yolk proteins of eggs from four individually housed captive budgerigars. Note that each female had a unique pattern of bands, which was repeatable within and between clutches. Egg yolk proteins were digested by trypsin and fragments visualized by silver staining. The migration positions of the molecular weight standards are indicated on the left. On the right we indicate which bands were used in the diagnosis of maternity.

For determining maternity in guira cuckoo eggs we did not compare gels that belonged to different groups, because we were interested only in identifying eggs belonging to females within groups. However, for calculating probability that two random females shared the same banding patterns, we compared bands across gels to determine presence or absence in different individuals. Only well-defined bands that could be scored reliably were used for maternal identification.

The validation of the protocol for guira cuckoos was made through PAGE analyses of yolk proteins in ovarian follicles from known females. Yellow, yolk-containing follicles, measuring 1.5–2.0 mm in diameter, were collected and processed according to Tyler (1993), with the following modifications: (i) four individual follicles from each of the three females were manually homogenized separately in distilled water (w:v = 1:100); (ii) the homogenate was then centrifuged at 1400 *g* for one min. Aliquots of 25 μL of supernatant were transferred to two new tubes and buffer only or enzyme and buffer were added to the tubes in the same proportion as for the egg yolk procedure. The subsequent protocol procedures for the follicle yolks were the same as for egg yolks (see above).

## Results

The protocol that best resolved the bands for both budgerigar and guira cuckoo egg and follicle yolks was trypsin digestion followed by resolution on a 12.0% separating gel. Although we could see slight differences among samples not treated with enzyme, they were not as visible as those we obtained when trypsin was added. Therefore we applied trypsin digestion of yolk proteins before PAGE.

Results for the budgerigar analyses showed identical banding patterns of yolk proteins for all of the eggs from the same females, including eggs in the same and in different clutches, and different patterns for all the eggs of distinct females (Fig. 1). For guira cuckoos, banding patterns for yolk proteins of follicles from the same female were identical, but different from follicles of distinct females.

During egg production, meiotic division starts just before ovulation, when the egg has already been yolked (Burley & Vadhera 1989); so the yolk proteins are gene products of a diploid situation, and maternally derived only. Thus, the identical banding patterns in eggs or follicles in the same female and the different pattern in eggs and follicles of different females can be used to identify eggs of unknown individual females. After this validation we applied the method to eggs of free-living guira cuckoo groups for further biological validation.

We monitored 27 nesting bouts from 14 active groups and 21 nesting bouts from 10 groups of guira cuckoos in 1998 and 1999, respectively. The number of adults seen at the nests ranged from one to eight individuals, with varying numbers of females (as determined by the electrophoresis of yolk proteins), and communal clutches in joint nests ranged from one to 19 eggs (Table 1). These were reduced through egg ejection, which averaged about 3.3 eggs/clutch for the two years. Of the total 171 eggs collected in 1998, 40 eggs were not suitable or available for electrophoresis because we found them during incubation, and 18 eggs were lost due to ejection (we found their eggshells broken on the ground). In 1999 we collected 126 eggs, of which 19 were not available for PAGE analyses (same reasons) and 25 were lost due to ejection. The majority of eggs for which we obtained no data (67% of 43 ejected eggs in both years) were first-laid eggs that were ejected promptly before the net was installed. Although we observed guira cukoos building new nests we could not disturb them by placing nets until the birds started laying, to avoid desertion. Finally, for the PAGE analyses we used 113 eggs from 17 nesting bouts in 12 active groups in 1998 and 82 eggs from 17 nesting bouts in 10 groups in 1999.

**Table 1** Characteristics of reproductive groups of guira cuckoos from central Brazil in 1998 and 1999

| Variable | 1998 | | 1999 | |
|---|---|---|---|---|
| | Mean ± SD (*n*) | Range | Mean ± SD (*n*) | Range |
| Number of adults/nest* | 4.4 ± 1.9 (21) | 1–7 | 4.7 ± 2.1 (16) | 1–8 |
| Number of females/nest† | 3.2 ± 2.0 (17) | 1–7 | 2.5 ± 1.4 (17) | 1–5 |
| Clutch size | 6.6 ± 5.5 (26) | 1–19 | 7.3 ± 3.4 (19) | 1–17 |
| Eggs tossed | 2.2 ± 2.5 (25) | 0–8 | 4.2 ± 4.0 (17) | 0–12 |

*Maximum number of (unbanded) adults seen simultaneously in each nest visit.
†Minimum number of laying females in the nesting bout, considering that maternity of some eggs could not be determined.

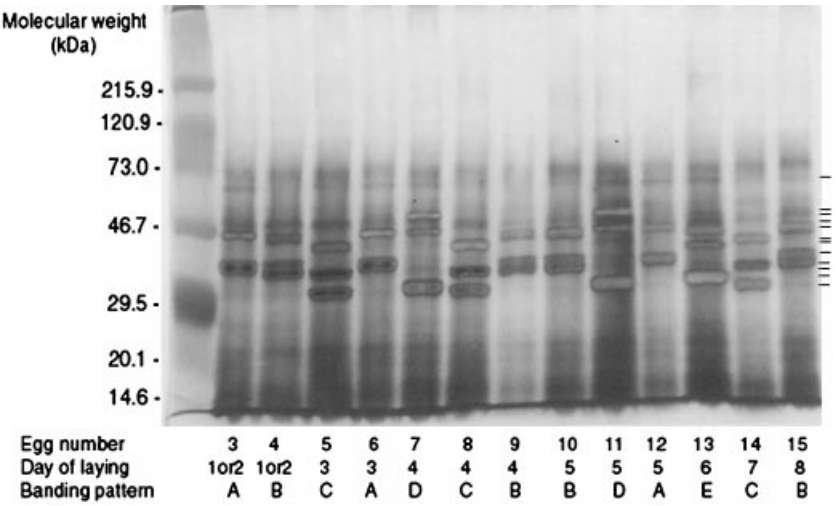


**Fig. 2** SDS-PAGE of yolk proteins of eggs from the second nesting bout of guira cuckoo group B8, in which 13 of 15 eggs laid were analysed. All eggs laid on each of days 3, 4 and 5 of the laying sequence had different banding patterns. Some identical banding patterns were repeated on different days. Egg yolk proteins were digested by trypsin and fragments visualized by silver-staining. The migration positions of the molecular weight standards are indicated on the left. On the right is indicated which bands were used in the diagnosis of maternity.

There were no cases of identical yolk protein banding patterns of eggs laid on the same day within any nest. This includes 22 cases where two eggs were laid in the same nest on the same day, and 12 cases where three eggs were laid in a single nest on the same day. For example, in Fig. 2 we show that all of the eggs laid within days 3, 4 and 5 in a joint nest have distinct banding patterns, indicating that on day 3 there were two females laying, and on days 4 or 5, three females contributed one egg each to the communal clutch. This substantiates our method further, because it is consistent with laying physiology which does not allow the laying of more than one egg per day by female birds (van Tienhoven 1983).

Identical banding patterns were always from eggs laid on different days of a nesting bout, which indicates that some females laid more than one egg in the laying sequence of the communal nest. This is also seen clearly in Fig. 2, wherein of the 13 total eggs analysed from that nesting bout, one female (B) laid four eggs, two females (A and C) each laid three eggs and one female (D) laid two eggs in different days. Only one female (E) laid a single egg in the total analysed.

Additionally, in guira cuckoo groups in which we monitored renestings we observed frequently that some banding patterns of yolk proteins were repeated in successive nesting bouts, while others were not. At least 13 females laid eggs in two nesting bouts of the same group and at least one female laid eggs in three renestings of the same group. For example, in group D5, female C laid eggs in all three nesting bouts of her group, two of which (1 and 3) are shown in Fig. 3. First-laid eggs lost due to tossing or eggs being incubated in complete clutches were not analysed, thus some laying females were possibly missed in our study.

Banding patterns of yolk proteins per communal clutch indicated that various females contribute eggs to nests, in repeated nesting bouts of each group. In rare instances, as many as seven females may lay simultaneously in a joint nest. In this exceptional case, seven females laid a total of 16 eggs: one female laid a single egg, three females each laid two eggs and three females each laid three eggs. The communal nature of reproduction in guira cuckoos is shown by the positive correlation between the number of laying females in joint nests with communal clutch size ($r^2 = 0.90$, $P < 0.001$, $n = 16$, Fig. 4), indicating a fairly equal partitioning of reproductive opportunities.

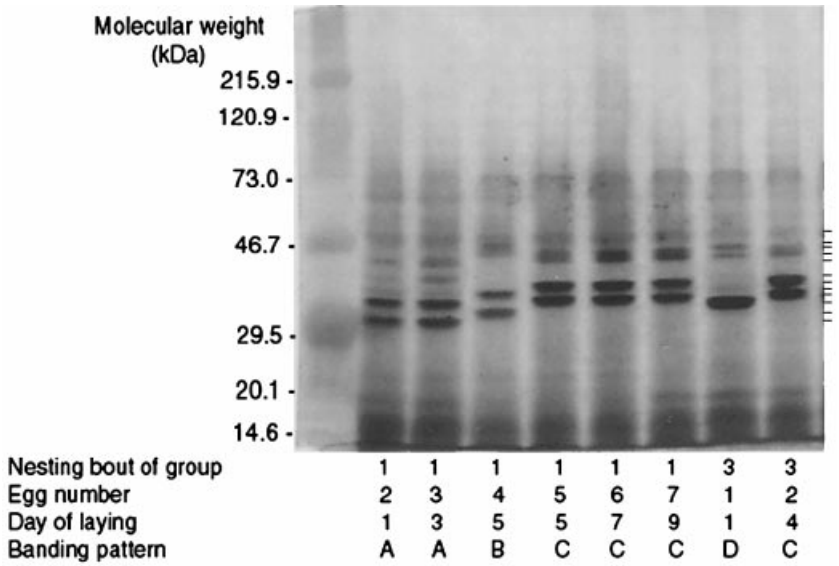


**Fig. 3** SDS-PAGE of yolk proteins of eggs from the first and third nesting bouts of the same guira cuckoo group (D5) indicating that the female represented by banding pattern C laid eggs in both bouts. Egg yolk proteins were digested by trypsin and fragments visualized by silver-staining. The migration positions of the molecular weight standards are indicated on the left. On the right we indicate which bands were used in the diagnosis of maternity.

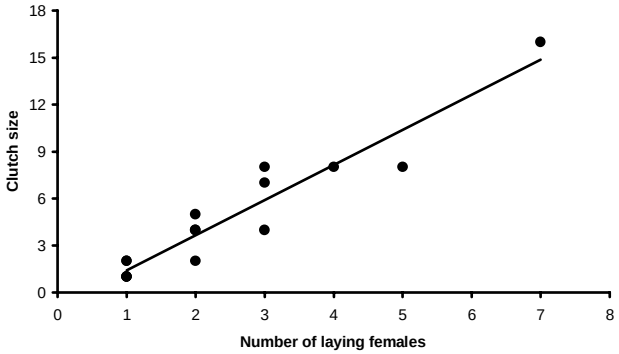


**Fig. 4** The relationship between the number of different females and communal clutch size for nests in which maternity of all eggs could be determined ($r^2 = 0.90$, $n = 16$, $P < 0.001$).

## Discussion

The validation of our protocol included known female budgerigar clutches, guira cuckoo egg follicles and biological aspects. Results suggest that yolk protein electrophoresis is an excellent tool for identifying maternity of eggs. The budgerigar and guira cuckoo analyses showed not only that our protocol was accurate in determining egg maternity for these birds due to sufficient female polymorphism for yolk proteins (Manwell & Baker 1975), but that with high likelihood the protocol can be used with other species.

Other supporting evidence for the protocol involves the scrutiny of banding patterns within and among nests of free-living guira cuckoo groups. First, all multiple eggs laid in a single nest on the same day had different patterns of proteins, practically nullifying the possibility that two or more females are represented by the same banding pattern. Second, eggs with the same pattern of bands were not laid on the same day but were repeated on different days, indicating that some females lay multiple eggs in successive days in a communal nest. Both results are also in agreement with physiological restraints that allow female birds to produce a single egg per day (van Tienhoven 1983).

Thirty-two (1998) and 16 (1999) females were involved in laying of multiple eggs in a single nest on the same day. For these cases, within each year, no identical banding patterns of yolk proteins occurred in nests of different groups. However, even if none of the eggs laid on the same day within a nest had identical banding patterns, there is a probability that two different females share the same banding pattern. We estimated this probability using Andersson & Åhlund's (2001) approach for albumen bands in common goldeneye duck eggs. This estimate uses expressions applied in individual identification in DNA fingerprinting studies (Jeffreys *et al*. 1985a,b; Burke & Bruford 1987; Georges *et al*. 1988). For this, we used eggs laid in nests at least 1 km apart, considerably reducing the chances that the same female laid eggs in more than one of these nests. We sampled a unique banding pattern from nests where there were no cases of multiple eggs laid within a 24-h period (three eggs laid in three nests total) plus two or more banding patterns from nests where multiple eggs were laid on the same day (24 eggs from nine groups). Because a female cannot lay more than one egg per day, multiple eggs found in joint nests within a period of 24 h clearly belonged to different females. We estimated $x$, the mean probability (across all bands) that a band in one individual also occurs in another random individual; $f$, the mean number of bands per individual (we eliminated bands not clearly defined for this estimate); and $q$, the allele (band) frequency,

estimated from $x = 2q - q^2$ (Jeffreys *et al*. 1985b). The results were: $x = 0.135$, $f = 4.81$, and $q = 0.070$. The mean probability that two random individuals will share identical banding patterns is thus $x^f = 0.135^{4.81} \approx 6.56 \times 10^{-5}$.

We also estimated this probability for the case of closely related females, although Quinn *et al*. (1994) showed that kinship among adults in guira cuckoo nests is relatively low. The probability of band sharing between siblings ($p_{sib}$) is $(1 + q - q^2)/(2 - q)$ or $(4 + 5q - 6q^2 + q^3)/4\,(2 - q)$ depending on whether the band variation is nonallelic or allelic in Hardy–Weinberg equilibrium (Jeffreys *et al*. 1985a). Using the first expression, two sisters will have a mean probability of $Ps = (p_{sib})^f = 0.552^{4.81} \approx 5.74 \times 10^{-2}$ of sharing an identical banding pattern, and using the second expression this probability is $0.560^{4.81} \approx 6.15 \times 10^{-2}$. Additionally, we estimated the probability of a mother and a daughter sharing the same banding pattern (as seen in DNA fingerprinting (Georges *et al*. 1988; Rabenold *et al*. 1991) and in protein fingerprinting (Andersson & Åhlund 2001)). Considering that the expected number of bands a female has inherited from her mother is $n_m = f(1 + q - q^2)/(2 - q)$, the expected number of bands she has inherited exclusively from her father is thus $n_p = f(1 - (1 + q - q^2)/(2 - q))$. For the daughter and mother to share an identical banding pattern, these $n_p$ bands inherited from the father must also be present in the mother. For each band, this probability is $x$. Thus, the probability that mother and daughter will have the same pattern of bands is $P_{m\text{-}d} = x^{n_p} = 0.135^{2.155} \approx 1.34 \times 10^{-2}$.

In our study there was one clutch to which seven females contributed eggs. Genetic analyses to determine maternity would require parental sampling for comparison of results, which would have been difficult to obtain given the high number of females and the difficulty in capturing them, and using only field observations the occurrence of three new eggs per day in this nest would have indicated only that a minimum of three females were laying when there were, in fact, seven females.

Some previous studies used allozymes analyses to discriminate between eggs of more than a single parasitic female of the same species laying in one nest (Fleischer 1985; Fleischer *et al*. 1985; Kendra *et al*. 1988; Fleischer & Smith 1992; Smyth *et al*. 1993). However, most of these studies could not reveal the exact number of females contributing eggs to nests, but only the minimum number. This occurred because detection of genetic differences was limited, and different females might present the same alleles and be mistakenly identified as the same female. This does not seem to occur in our analysis because every known budgerigar or guira cuckoo female had a particular banding pattern of yolk proteins. Andersson & Åhlund (2001) also found unique patterns of bands for egg white proteins of eggs from known common goldeneye duck females.

In this study we collected whole eggs (ejected as well as retained in the nests) because egg yolk, egg white and eggshell masses were required for other objectives. However, if only the identification of egg maternity is desirable, small yolk samples (mg) may be obtained by puncturing the eggshell with a needle to sample the yolk without embryonic damage, and eggs can be returned to the nest to continue development (see Schwabl 1993 for details).

Although guira cuckoo groups contain a number of females, they do not always contribute to each nesting bout of the group, and some females contribute more eggs to different clutches than do other females. This is somewhat different from what has been proposed in the literature for the groove-billed ani (*Crotophaga sulcirostris*), another crotophagine communal breeder, where females in the group are reported to contribute eggs to all nesting bouts (Vehrencamp 1977).

Thus, the biochemical technique proposed is the best current method to identify individual clutches in guira cuckoos and may be broadly applicable in other ornithological studies, especially when the use of genetic analyses is impossible. As in 'protein fingerprinting' of egg white, this technique has several advantages over DNA methods, such as the ease in application and low cost involved (Andersson & Åhlund 2001). It can elucidate individual reproductive benefits and strategies of females in communal and cooperative breeding species, polygynous mating systems with joint nests, and in cases of intraspecific nest parasitism.

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## References

Andersson M, Åhlund M (2001) Protein fingerprinting: a new technique reveals extensive conspecific brood parasitism. *Ecology*, **82**, 1433–1442.

Andersson M, Eriksson MOG (1982) Nest parasitism in goldeneyes *Bucephala clangula*: some evolutionary aspects. *American Naturalist*, **120**, 1–16.

Brown CR (1984) Laying eggs in a neighbor's nest: benefit and cost of colonial nesting in swallows. *Science*, **224**, 518–519.

Brown CR, Brown MB (1988) Genetic evidence of multiple parentage in broods of cliff swallows. *Behavioral Ecology and Sociobiology*, **23**, 379–387.

Brown JL (1987) *Helping and Communal Breeding in Birds*. Princeton University Press, Princeton, NJ.

Brown JL, Brown EL (1990) Mexican jays: uncooperative breeding. In: *Cooperative Breeding in Birds: Long-Term Studies of Ecology and Behavior* (eds Stacey PB, Koenig WD), pp. 269–288. Cambridge University Press, Cambridge.

Burke T, Bruford MW (1987) DNA fingerprinting in birds. *Nature*, **327**, 149–152.

Burley RW, Vadhera DV (1989) *The Avian Egg: Chemistry and Biology*. John Wiley, New York.

Craig JL (1979) Habitat variation in the social organization of a communal gallinule, the Pukeko (*Porphyrio porphyrio*). *Behavioral Ecology and Sociobiology*, **5**, 331–358.

Davis DE (1942) The phylogeny of social nesting habits in the Crotophaginae. *Quarterly Review of Biology*, **17**, 115–134.

Fleischer RC (1985) A new technique to identify and assess the dispersion of eggs of individual brood parasites. *Behavioral Ecology and Sociobiology*, **17**, 91–99.

Fleischer RC, Murphy MT, Hunt LE (1985) Clutch size increase and intraspecific brood parasitism in the yellow-billed cuckoo. *Wilson Bulletin*, **97**, 125–127.

Fleischer RC, Smith NG (1992) Giant cowbird eggs in the nests of two icterid hosts: the use of morphology and electrophoretic variants to identify individuals and species. *Condor*, **94**, 572–578.

Georges M, Lequarré A-S, Castelli M, Hanset R, Vassart G (1988) DNA fingerprinting in domestic animals using four different minisatellite probes. *Cytogenetics and Cell Genetics*, **47**, 127–131.

Gowaty PA, Karlin AA (1984) Multiple maternity and paternity in single broods of apparently monogamous eastern bluebirds (*Sialia sialis*). *Behavioral Ecology and Sociobiology*, **15**, 91–95.

Jeffreys AJ, Brookfield JFY, Semenoff R (1985a) Positive identification of an immigration test-case using DNA fingerprints. *Nature*, **317**, 818–819.

Jeffreys AJ, Wilson V, Thein SL (1985b) Individual-specific 'fingerprints' of human DNA. *Nature*, **316**, 76–79.

Kendra PE, Roth RR, Tallamy DW (1988) Conspecific brood parasitism in the house sparrow. *Wilson Bulletin*, **100**, 80–90.

Koenig WD (1981) Reproductive success, group size, and the evolution of cooperative breeding in the acorn woodpecker. *American Naturalist*, **117**, 421–443.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lahti DC, Lahti AR (2002) How precise is egg discrimination in weaverbirds? *Animal Behaviour*, **63**, 1135–1142.

Macedo RH (1992) Reproductive patterns and social organization of the communal guira cuckoo (*Guira guira*) in central Brazil. *Auk*, **109**, 786–799.

Macedo RH (1994) Inequities in parental effort and costs of communal breeding in the guira cuckoo. *Neotropical Ornithology*, **5**, 79–90.

Macedo RH, Bianchi CA (1997a) Communal breeding in tropical guira cuckoos *Guira guira*: sociality in the absence of a saturated habitat. *Journal of Avian Biology*, **28**, 207–215.

Macedo RHF, Bianchi CA (1997b) When birds go bad: circumstantial evidence for infanticide in the communal South American guira cuckoo. *Ethology, Ecology and Evolution*, **9**, 45–54.

Macedo RHF, Cariello M, Muniz L (2001) Context and frequency of infanticide in communally breeding guira cuckoos. *Condor*, **103**, 170–175.

Manwell C, Baker CMA (1975) Molecular genetics of avian proteins XIII. Protein polymorphism in three species of Australian passerines. *Australian Journal of Biological Sciences*, **28**, 545–557.

McKitrick MC (1990) Genetic evidence for multiple parentage in eastern kingbirds (*Tyrannus tyrannus*). *Behavioral Ecology and Sociobiology*, **26**, 149–155.

Melo C (1997) *Reprodução Comunitária Em Guira Guira Gmelin 1788 (Cuculidae, Aves): Aspectos Eto-Ecológicos*. Master's Thesis, Universidade de Brasília, Brazil.

Melo C, Macedo RHF (1997) Mortalidade em ninhadas de *Guira guira* (Cuculidae): competição por recursos? *Ararajuba*, **5**, 49–56.

Møller AP (1987) Intraspecific nest parasitism and anti-parasite behaviour in swallows, *Hirundo rustica*. *Animal Behaviour*, **35**, 247–254.

Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry*, **117**, 307–310.

Quinn JS, Macedo R, White BN (1994) Genetic relatedness of communally-breeding guira cuckoos. *Animal Behaviour*, **47**, 515–529.

Rabenold P, Rabenold KN, Piper WH, Decker MD, Haydock J (1991) Using DNA fingerprinting to assess kinship and genetic structure in avian populations. In: *The Unity of Evolutionary Biology* (ed. Dudley EC), pp. 611–620. Dioscorides, Portland.

Schwabl H (1993) Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Sciences of the USA*, **90**, 11446–11450.

Sick H (1997) *Ornitologia Brasileira*. Nova Fronteira, Rio de Janeiro.

Smyth AP, Orr BK, Fleischer RC (1993) Electrophoretic variants of egg white transferrin indicate a low rate of intraspecific brood parasitism in colonial cliff swallows in the Sierra Nevada, California. *Behavioral Ecology and Sociobiology*, **32**, 79–84.

van Tienhoven A (1983) *Reproductive Physiology of Vertebrates*, 2nd edn. Cornell University Press, Ithaca/London.

Tyler C (1993) Electrophoretic patterns of yolk proteins throughout ovarian development and their relationship to vitellogenin in the rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology Part B*, **106**, 321–329.

Vehrencamp SL (1977) Relative fecundity and parental effort in communally nestings anis, *Crotophaga sulcirostris*. *Science*, **197**, 403–405.

Vehrencamp SL, Bowen BS, Koford RR (1986) Breeding roles and pairing patterns within communal groups of groove-billed anis. *Animal Behaviour*, **34**, 347–366.

Wrege PH, Emlen ST (1987) Biochemical determination of parental uncertainty in white-fronted bee-eaters. *Behavioral Ecology and Sociobiology*, **20**, 153–160.

Yom-Tov Y (1980) Intraspecific nest parasitism in birds. *Biological Review*, **55**, 93–108.

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Mariana Cariello, a doctoral student at Universidade de Brasilia, Brazil, focused on maternal reproductive investment in guira cuckoos, a study within an ongoing project on the behavioural ecology of guira cuckoos in Brazil. She conducted the electrophoresis within a collaborative project between H. Schwabl and R.H. Macedo. The field project was coordinated by R.H. Macedo while H. Schwabl coordinated the development of the yolk protein electrophoresis method. R. Lee, a professor in environmental physiology at Washington State University, has interests in the application of biochemical/molecular approaches to ecological investigations and provided expertise with the fine-tuning of the analyses.

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