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ORIGINAL PAPER

# Eggshell spotting in brood parasitic shiny cowbirds (*Molothrus bonariensis*) is not linked to the female sex chromosome

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**Abstract** For avian brood parasites in which individual females are host-specialists, the arms race between hosts and parasites has favored egg color polymorphism in the parasite, with female lineages laying mimetic eggs that resemble those of the host species they parasitize. Female sex-linked inheritance of egg color fosters evolutionary stability of egg polymorphism if female lineages show both consistent eggshell color and host use. This co-evolutionary relationship is unlikely to occur if individual brood parasites use different hosts or if egg color is not maternally inherited. The shiny cowbird (*Molothrus bonariensis*) is an extreme generalist brood parasite that shows a very high degree of egg polymorphism. We tested whether egg spotting in this species has female sex-linked inheritance. If genetic factors controlling the expression of egg spotting were present on the female-specific W chromosome, we expected co-segregation between spotting patterns and mtDNA haplotypes, as both W and mtDNA are maternally inherited. In contrast to the known maternal inheritance of spotting patterns in great tits, we found no associations between eggshell spotting and mtDNA haplotypes, which suggests that eggshell spotting is not maternally inherited in this cowbird species.

**Keywords** Brood parasitism · Eggshell spotting · *Molothrus bonariensis* · mtDNA · Shiny cowbird

## Introduction

The color and pattern of avian eggshells vary considerably among and within species (Underwood and Sealy 2002). Interactions between hosts and brood parasites have been one of the strongest forces of selection on variation in egg appearance (e.g., Øien et al. 1995; Soler and Møller 1996; Lahti and Lahti 2002). In some of the best studied host–parasite systems involving parasitic cuckoos, the arms race between hosts that reject non-mimetic eggs and parasites that evolve egg coloration mimicking that of its hosts (Gibbs et al. 2000; Honza et al. 2001) has selected for egg polymorphism in the parasites, in which female host-specific races (gentes) lay mimetic eggs that resemble those of the host they parasitize (Brooke and Davies 1988; Moksnes and Røskaft 1995). Affected hosts have in turn evolved decreased intraclutch and increased interclutch egg variability to distinguish their eggs from those of the parasites (Victoria 1972; Davies and Brooke 1989; Soler et al. 2000; Stokke et al. 2002; Avilés and Møller 2003; Lahti 2005; Antonov et al. 2006; but see Avilés et al. 2004, Cherry et al. 2007). This co-evolutionary process is unlikely to occur in systems involving brood parasites where individual females use many hosts (Stokke et al. 2002), as each such female will parasitize nests of many hosts with variable eggshell colors.

The shiny cowbird *Molothrus bonariensis* is an extreme generalist brood parasite that has been documented using

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more than 240 species as hosts (Ortega 1998). Shiny cowbirds show high egg polymorphism, with eggs varying from immaculate to heavily spotted and with ground colors including pure white, light cream, light bluish white, light greenish white, dark cream, or light brown (Hudson 1874; Friedmann 1929; Ortega 1998). Within this range of variation in spotting patterns, eggs can be roughly classified in three categories: white immaculate, intermediate, and spotted. Immaculate eggs are pure white without any spots or markings whatsoever, intermediate eggs have very few fine spots and appear white from a distance, and spotted eggs vary enormously in the ground color and the markings (Hudson 1874; Friedmann 1929; Fig. 1). White immaculate eggs are present in eastern Argentina, Uruguay, and southeastern Brazil where their frequency can be as high as 50% (Fig. 2), but they appear to be absent elsewhere in South America, except for SW Ecuador (Marchant 1960), and in the regions colonized by shiny cowbirds' expansion during the 20th century (Caribbean islands; Post and Wiley 1977). Closely related parasitic species including the bronzed cowbird *Molothrus aeneus* (Harrison 1978; Peer et al. 2002) and the giant cowbird *Molothrus orizivorus* (Haverschmidt 1966; Fleischer and Smith 1992) also lay immaculate eggs, whereas the screaming cowbird *Molothrus rufoaxillaris* and the brown-headed cowbird *Molothrus ater* lay only spotted eggs (Ortega 1998).

Previous studies have found diverse evidence of genetic control of egg color. Collias (1993) found that individual village weaver *Ploceus cucullatus* females lay just one egg type throughout their life, and that the Mendelian inheritance of the eggs' ground coloring was best explained by two autosomal loci. Autosomal inheritance of egg color was also suggested for the domestic chicken *Gallus domesticus* (Hutt 1949). In contrast, Gosler et al. (2000) showed that eggshell spotting patterns of daughters in the great tit *Parus major* resembled those of their mothers and maternal grandmothers but not of their paternal grandmothers, which is consistent with female sex-linked inheritance. Inheritance of egg coloration linked to the female W chromosome also appears to account for the evolutionary stability of egg polymorphism in the common cuckoo *Cuculus canorus* (Marchetti et al. 1998; Gibbs et al. 2000), where females of a host-specific lineage share their mtDNA haplotype and the coloration of their eggs, which mimic those of the host they parasitize. Female brown-headed cowbirds lay eggs with a consistent color pattern (Dufty 1983, Fleischer 1985), but little is known about the inheritance of egg coloration in the cowbirds. Kilner (2006) suggested that the shiny cowbird egg spotting is controlled by an autosomal locus with two alleles, one for spotting and one for no spotting, with heterozygotes laying eggs with the intermediate phenotype.

The aim of our study was to test the hypothesis of female sex-linked inheritance of egg color variation in shiny cowbirds by analyzing whether spotting in this species is associated with female-specific genetic markers. If genetic

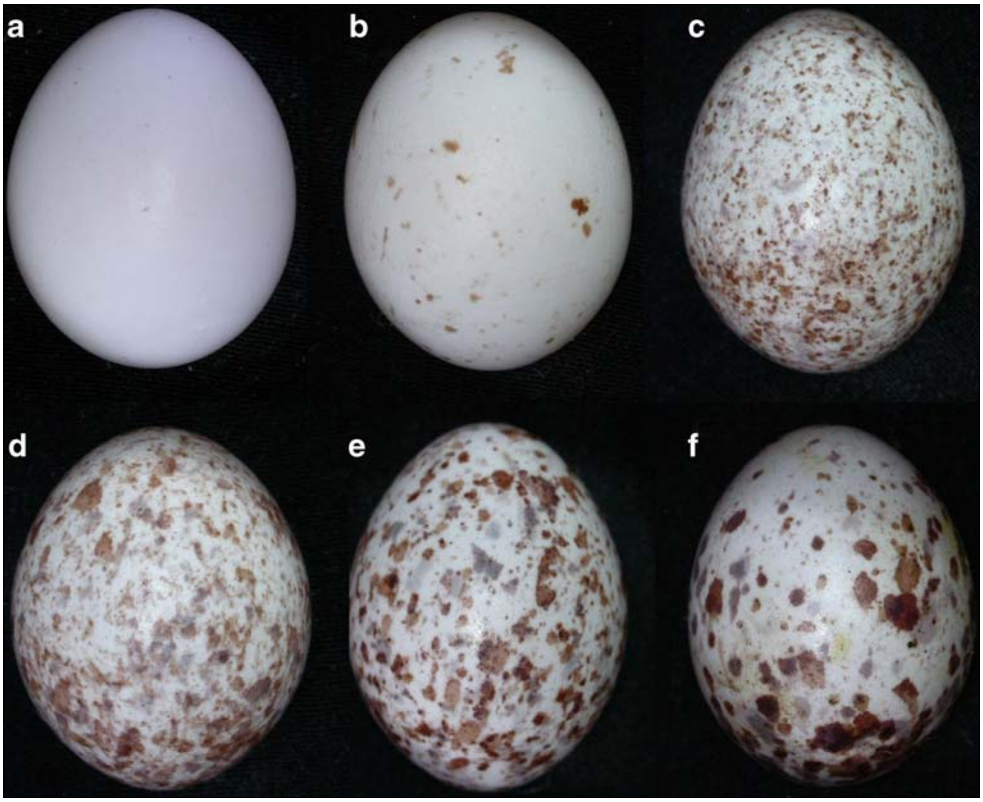

**Fig. 1** Photograph illustrating shiny cowbird eggshell color variation. **a** White immaculate, **b** intermediate, **c–f** spotted

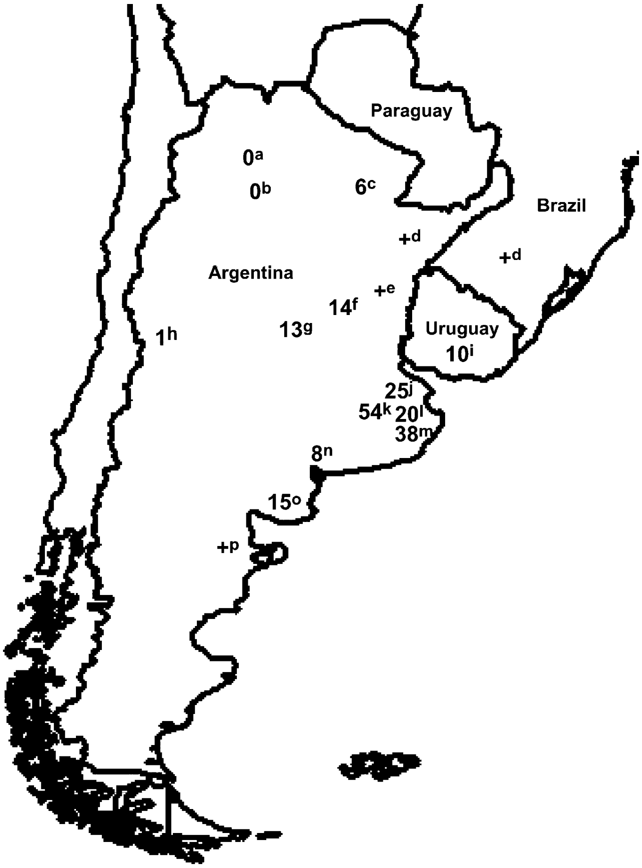

**Fig. 2** Known distribution of shiny cowbird egg morphs across southern South America. *Numbers* indicate frequencies of white immaculate eggs; *plus sign* indicates that white eggs are present but at unknown frequency. *Letters* indicate the locality and source of the data. *a* Salta (Miller 1917; Hoy and Ottow 1964), *b* Tucumán (King 1973), *c* Formosa (Di Giacomo pers. comm.), *d* Corrientes (Friedmann 1929), *e* Entre Ríos (Friedmann 1929), *f* Santa Fé (De la Peña pers. comm.), *g* Córdoba (Salvador 1983), *h* Mendoza (Astié and Reboreda 2005), *i* Uruguay (Lereté pers. comm.), *j* Magdalena, Buenos Aires (Tuero et al. 2007), ***k*** Lobos, Buenos Aires (Fraga 1978), *l* General Lavalle, Buenos Aires (Massoni and Reboreda 1998), *m* General Lavalle, Buenos Aires (Lyon 1997), *n* Tornsquist, Buenos Aires (Cozzani pers. comm.), *o* Rio Negro (Salvador and Salvador 1984), *p* Chubut (Salvador and Salvador 1984)

factors controlling the expression of egg spotting were present on the female-specific W chromosome, we expected co-segregation between spotting and mtDNA haplotypes, as the W chromosome and mtDNA are both clonally and maternally transmitted in birds (Berlin and Ellegren 2001). Thus, for each mitochondrial haplotype, we expected to find eggs of a consistent spotting pattern. In contrast, if genetic factors controlling eggshell spotting are not strictly female sex linked, we expected to find no such association. Although the co-segregation of mtDNA molecular markers and female sex-linked characters have been studied in other taxa (e.g., wing color in butterflies; Andolfatto et al. 2003), this is its first application to study the inheritance of avian eggshell spotting.

## Materials and methods

### Cowbird samples

We collected cowbird eggs during the breeding seasons (October–January) of 2002–2003, 2003–2004, and 2004–2005 at three different locations in Buenos Aires Province, Argentina: Magdalena (35° 08′ S, 57° 23′ W), General Lavalle (36° 26′ S, 56° 25′ W), and Chascomús (35° 34′ S, 58° 01′ W). These locations are 70 to 150 km apart from each other. Eggs were collected from nests of four host species: southern house wren *Troglodytes musculus* ($n$=30), rufous-collared sparrow *Zonotrichia capensis* ($n$=17), chalk-browed mockingbird *Mimus saturninus* ($n$=24), and brown-and-yellow marshbird *Pseudoleistes virescens* ($n$=25). Eggs were collected from house wren nests built in artificial nest boxes, whereas for the other three hosts, all were collected from natural nests.

Freshly laid eggs were incubated at 37.5±1°C for 48 h to obtain some embryonic development prior to DNA extraction (Strausberger and Ashley 2001). Eggs found following the onset of incubation were processed without artificial incubation. Embryonic tissue was extracted from the eggs and stored in DMSO buffer (20% *v*/*v* DMSO, 250 mM EDTA, NaCl) for subsequent genetic analysis. Eggs were assigned by human observers into one of three color morphs: white immaculate, intermediate, and spotted (Mason 1986; Fig. 1). For genetic analyses, intermediate and spotted eggs were pooled into one category because both egg morphs contain pigmentation beyond the background color, although to a different degree, and Gosler et al. (2005) showed that in great tits *P. major*, the intensity and size of spots were heritable but not their overall distribution on the eggshell. Therefore, in our study, we tested if the presence of spots on the eggshell is maternally inherited but do not analyze spotting patterns in detail. To avoid categorization biases, we also repeated the analysis after excluding eggs of the intermediate morph ($N$=9).

### mtDNA analyses

To assess mtDNA variation, we sequenced a 1,120-base-pair fragment of the control region, using two sets of primers: GSH-12 s and GSL-GLU (Gibbs et al. 1997) and MBO-L1 and MBO-H2 (Mahler et al. 2007). DNA was extracted from tissue samples with Eppendorf and QIAGEN extraction kits. PCR amplifications were conducted as in Mahler et al. (2007). Amplified products were sequenced on an Applied Biosystems Model 3100 Genetic Analyzer using ABI Big Dye™ Terminator Chemistry. Nucleotide sequences have been deposited in GeneBank under accession numbers DQ683547–DQ683561.

### Data analysis

Sequences were compiled in Sequencher v.3.1.1 (Genecodes Corp.) and Bioedit v.7.0.5.3 software (Hall 1999) and aligned using Clustal W (Thompson et al. 1994). Phylogenetic relationships among mtDNA haplotypes were inferred using maximum parsimony, as implemented in TNT (Goloboff et al. 2003). Exact searches were performed using the "implicit enumeration" option.

We tested for genetic differentiation between egg morphs using Arlequin v.2.0 (Schneider et al. 2000). We used analysis of molecular variance (AMOVA; Excoffier et al. 1992), which partitions total variance into within versus between group components (Hudson et al. 1992) through $\Phi_{ST}$ that takes into account both haplotype frequencies and molecular pairwise differences. The average number of nucleotide differences between sequences was estimated using the Kimura 2-parameter model of nucleotide substitution. Significance levels were determined using permutation procedures as implemented in Arlequin. Because specific algorithms for power estimation of AMOVA are not available, we tested the power of our AMOVA using simulations (Buonacrossi et al. 2001; Nievergelt et al. 2007). We generated simulations of 12 white eggs and 82 spotted eggs on the haplotype network, leaving the frequency of each haplotype fixed and varying the number of spotted or white eggs within each haplotype. This procedure was repeated for situations of one to four independent origins of white eggs. For one gain (without a posterior transition to spotted eggs), 21 simulations were possible, whereas for two, three, and four gains we generated 100 color morph distributions, including different haplotype combinations showing white eggs and different frequencies of white eggs per haplotype. We calculated $\Phi_{ST}$ and its associated significance level for each simulation and counted the number of significant simulations at $\alpha=0.05$.

The statistical analysis was performed on all samples and repeated for smaller subsets of samples that accounted for possible biases due to differences between sampling locality or host species. First, sampling locality might generate a bias in haplotype frequency distribution if shiny cowbird populations are isolated between sampling areas, and females of one particular haplotype lay white eggs in one locality but spotted ones in another locality. In this case, although egg color may be linked to mtDNA haplotype in each locality, a global analysis would yield no association. Therefore, we repeated the test for each locality separately. Second, host species might generate a bias if shiny cowbird females are host specific, and hosts have different egg-rejection behavior. The hosts included in this study vary in the rejection of shiny cowbird eggs, and whereas white eggs are ejected from the nest by chalk-browed mockingbirds (Fraga 1985; Sackmann and Reboreda 2003) and brown-and-yellow marshbirds (Mermoz and Reboreda 1994) within the first 24–48 h, this egg morph is accepted by house wrens (Tuero et al. 2007) and rufous-collared sparrows (Fraga 1978). Differential rejection of egg morphs might create an indirect association of white eggs with mtDNA haplotype stemming from an association of mtDNA haplotype with host use. Therefore, we repeated the analysis including only samples from the two host species that accept all egg morphs.

## Results

We obtained control region sequences from 94 cowbird eggs found in nests of four different hosts at three locations in Buenos Aires Province (Table 1). The sequences showed a total of 17 variable sites and resulted in 15 different haplotypes. Phylogenetic relationships among haplotypes yielded four most parsimonious networks. Figure 3 shows a network representing one of these unrooted trees; the alternative topologies differed in the relative position of a few haplotypes. We found three frequent haplotypes (H1, H2, and H7), three less frequent haplotypes (H4, H5, and

**Table 1** Number of shiny cowbird eggs per host and locality collected during 3 breeding seasons (October–January 2002–2003, 2003–2004, and 2004–2005)

| Host | Locality | | | Total |
|---|---|---|---|---|
| | Magdalena | General Lavalle | Chascomús | |
| Southern house wren | 16 | 1 | 5 | 22 |
| | 5 | 1 | 0 | 6 |
| Rufous-collared sparrow | 0 | 16 | 0 | 16 |
| | 0 | 1 | 0 | 1 |
| Chalk-browed mockingbird | 20 | 1 | 1 | 22 |
| | 2 | 0 | 0 | 2 |
| Brown-and-yellow marshbird | 0 | 22 | 0 | 22 |
| | 0 | 3 | 0 | 3 |
| Total | 36 | 40 | 6 | 82 |
| | 7 | 5 | 0 | 12 |

*Upper rows* Spotted eggs, *lower rows* white eggs

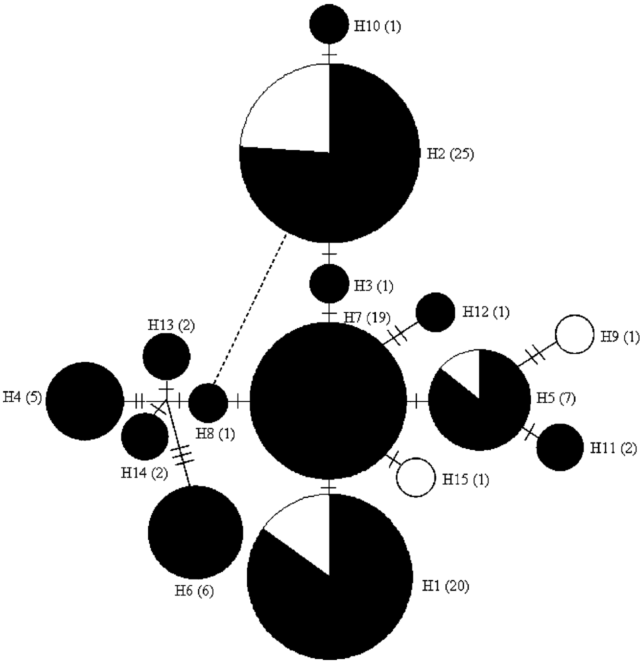

**Fig. 3** Unrooted, maximum parsimony network for 15 shiny cowbird haplotypes (*H1–H15*). *Numbers in parentheses* show the number of samples per haplotype. *Circle size* is proportional to haplotype frequency and *hatchmarks* show the number of nucleotide differences. *Shading* indicates the egg morph associated with each haplotype (*black* spotted, *white* white immaculate). Alternative connections defining other equally parsimonious trees are shown by *dotted lines*

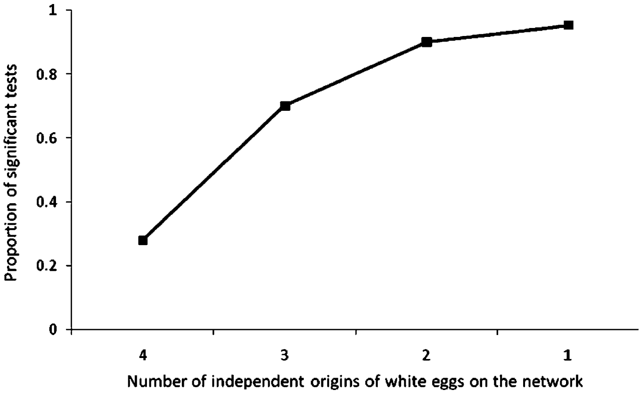

**Fig. 4** Proportion of simulations with $\Phi_{\mathrm{ST}}$ values with $P<0.05$ for distributions of 12 white eggs and 82 spotted eggs on the network, considering four to one independent origins of white eggs

H6), and nine rare haplotypes that were only found in one or two individuals. White eggs were found associated to five different haplotypes (Fig. 3), and its presence can be explained by at least four independent origins. Frequent haplotypes, except H7, were found in eggs of different spotting types (Fig. 3), and statistical tests showed that haplotype frequency distributions between the two types of egg morphs did not differ ($\Phi_{\mathrm{ST}}=0.01$; $P=0.24$). Females carrying the same mtDNA haplotype lay eggs of different coloration, which does not support the female sex-linked co-segregation model. Results did not vary when the eggs of the intermediate morph were excluded ($\Phi_{\mathrm{ST}}=0.03$; $P=0.15$), when localities were analyzed separately (General Lavalle: $\Phi_{\mathrm{ST}}=0.04$; $P=0.16$; Magdalena: $\Phi_{\mathrm{ST}}=0.09$; $P=0.07$) or when the analysis was restricted to only house wren and rufous-collared sparrow samples ($\Phi_{\mathrm{ST}}=0.004$; $P=0.33$).

Simulations for power analysis showed that significant results increase as independent gains of white eggs decrease (Fig. 4). Significance was strongly determined by very few white eggs in frequent haplotypes (H1, H2, and H7) and by less frequent haplotypes (H4, H5 and H6) showing only white eggs. Even distribution of white eggs in different haplotypes of the network, as was found in our case (Fig. 3), mostly yielded non-significant results. Fewer independent gains of white eggs, as expected by the co-segregation model, imply an uneven distribution of this egg morph on the network and thus more significant results.

## Discussion

We found no association between mtDNA haplotypes and presence of spotting in shiny cowbird's eggshells. Therefore, our results do not support the co-segregation model that would be expected if eggshell spotting in shiny cowbirds is maternally inherited via the W sex chromosome.

Genealogies of mtDNA and the W chromosome are completely concordant in birds (Berlin and Ellegren 2001). Therefore, if control of egg spotting were linked to the female sex chromosome, each haplotype should be associated with one egg morph, and related haplotypes should share egg-morph type, leading to the evolution of different egg-morph lineages. This pattern is expected as mutation rates of mtDNA, and specially the control region, are higher than mutation rates of typical nuclear genes (Avise 2004); furthermore, mutation rates of the avian W chromosome are substantially lower than those of autosomes and the Z chromosome (Axelsson et al. 2004), and variability in this chromosome is manifold lower than in autosomes (Montell et al. 2001; Berlin and Ellegren 2004). Our haplotype network shows that white eggs are associated with five of the sampled haplotypes, which are distributed across the network; three of these haplotypes were associated with both spotted and non-spotted eggs (Fig. 3). Although some gene(s) related to eggshell color may be located in the female-specific W chromosome with another autosomal epistatic gene controlling for the spotting pattern of the eggs, the primary inheritance of eggshell patterning appears not to be female sex linked.

An alternative possibility to genetic control of egg color is that egg spotting polymorphism arises as a consequence of environmental differences. Cherry and Bennett (2001) proposed that the egg color matching of the red-chested cuckoo (*Cuculus solitarius*) and its hosts could be a consequence of spatial autocorrelation of diet of cuckoos and hosts, and more recently, Avilés et al. (2007) found that rainfall and temperature were associated with reed warbler (*Acrocephalus scirpaceus*) and cuckoo (*C. canorus*) egg color, influencing parasite–host egg matching. However, it seems unlikely that differences in diet, rainfall, or temperature could explain the distribution and frequencies of shiny cowbird white immaculate eggs (see Fig. 2), as immaculate and spotted eggs are commonly found at the same times, locations, and hosts.

It has been proposed that egg polymorphism in shiny cowbirds could be associated with acceptance status of local hosts (Friedmann et al. 1977; Ortega 1998; Kilner 2006). In the area where immaculate eggs are most frequent, some shiny cowbird hosts accept both egg morphs (Mason 1986; Lyon 1997; Massoni and Reboreda 1998; Tuero et al. 2007), others eject white and accept spotted eggs (Fraga 1985; Mason 1986; Mermoz and Reboreda 1994; Sackmann and Reboreda 2003; Astié and Reboreda 2005), and there is one host that ejects eggs of both morphs if they are smaller than its own eggs (Mason and Rothstein 1986). However, there are no known hosts that eject shiny cowbird spotted eggs using color as a cue, precluding the possibility of a balanced polymorphism as has been suggested by Friedmann et al. (1977). Therefore, it seems unlikely that acceptance status of local hosts could explain frequencies of white eggs as high as 50%, which would also exclude albinism as a possible explanation (Gross 1968).

In sum, our results suggest that egg spotting in shiny cowbirds is not linked to the female-specific W chromosome, as has been suggested for other bird species (Gosler et al. 2000; Gibbs et al. 2000). This lack of W linkage would explain why female shiny cowbirds persist in parasitizing hosts that reject the white morph (i.e., Mermoz and Reboreda 1999), because, unless there is assortative mating by egg type, females cannot anticipate the appearance of their own eggs. The origin and maintenance of the high frequencies of immaculate white eggs in some areas of shiny cowbird distribution deserves further research.

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