



Genetic patterns of repeat and multiple parasitism by screaming cowbirds, a specialist brood parasite

Cynthia A. Ursino^{a, b, **}, Meghan J. Strong^a, Juan C. Reboreda^b, Christina Riehl^{a, *}

^a Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ, U.S.A.

^b Departamento de Ecología, Genética y Evolución, IEGEBA, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina

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Avian brood parasites lay their eggs in the nests of other species, leaving the hosts to care for the parasitic offspring. The bookkeeping hypothesis predicts that, in order to reduce competition between parasitic nestlings, female parasites should keep a mental inventory of host nests that they have already parasitized and avoid laying multiple eggs in the same host nest. However, selection against repeat parasitism should be weaker when host nests are limited, or when hosts are able to rear more than one parasitic nestling. Here we use microsatellite genotyping of parasitic eggs to test whether female screaming cowbirds, *Molothrus rufoaxillaris*, avoid repeatedly parasitizing nests of their primary host, the greyish baywing, *Agelaioides badius*, in Argentina. Parasitism rates were extremely high (96.5% of 57 host clutches were parasitized with an average of 5.7 cowbird eggs each), indicating that host nests are limited. Although eggs laid by the same female showed moderate spatiotemporal clustering, individual females rarely laid more than one egg in the same host clutch (2 of 57 clutches, 26 of which contained multiple genotyped cowbird eggs). Females were much more likely to lay subsequent eggs in different host nests than to return to the same host nest. We found no evidence for kin structure among female cowbirds parasitizing the same host nest, which were no more closely related than chance would predict. These results suggest that female screaming cowbirds frequently lay eggs in host nests that have already been parasitized by unrelated females. However, they typically lay just one egg per host clutch, even though greyish baywings are capable of rearing several nestlings. Since screaming cowbird laying is often poorly synchronized with that of their host, avoidance of repeat parasitism may be adaptive if it allows females to spread the risk of failure among multiple host nests.

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Avian brood parasites lay their eggs in the nests of other species, leaving the hosts to provide parental care to the parasitic offspring. Female parasites must therefore locate potential host nests, monitor them to assess their reproductive status and synchronize their egg laying with that of the host (Reboreda, Fiorini, De Mársico, Glog, & Scardamaglia, 2017; Spottiswoode, Kilner, & Davies, 2012). Furthermore, several authors have proposed that in order to reduce competition between parasitic nestlings, females should avoid laying multiple eggs in the same host nest (repeat parasitism; Ellison, Sealy, & Gibbs, 2006; Glog, Fiorini, Reboreda, & Kacelnik, 2014), or in nests that have already been parasitized by other

females (multiple parasitism; Hahn, Sedgwick, Painter, & Casna, 1999; McLaren, Woolfenden, Gibbs, & Sealy, 2003).

The costs of repeat and multiple parasitism vary across species, depending on the virulence of the parasite and the ability of the host to raise more than one parasitic nestling. In some species, including honeyguides (*Indicator* spp.) and several cuckoos (*Cuculus* spp., *Tapera naevia*), the parasitic nestling kills all other eggs and/or nestlings in the brood, so only one nestling can survive (Kilner, 2005). Selection against repeat parasitism may also be strong in situations where parasites destroy other eggs in the host nest prior to laying their own, such that females that lay repeatedly in the same nest risk damaging their own previously laid eggs (Fiorini, Glog, Kacelnik, & Reboreda, 2014; Glog et al., 2014; Scardamaglia, Fiorini, Kacelnik, & Reboreda, 2017); or where the host species is capable of raising just one or two nestlings, such that nestlings within the brood experience intense competition for food (Goguen, Curson, & Mathews, 2011; Spottiswoode et al., 2012). By contrast, repeat parasitism should be less costly (and selection

* Correspondence: C. Riehl, Department of Ecology and Evolutionary Biology, Princeton University, 106A Guyot Hall, Princeton, NJ, 08544, U.S.A.

** Correspondence: C. Ursino, Department of Ecology and Evolutionary Biology, Princeton University, 106A Guyot Hall, Princeton, NJ, 08544, U.S.A.

E-mail addresses: cursino@princeton.edu (C. A. Ursino), criehl@princeton.edu (C. Riehl).

against it correspondingly weaker) in situations where parasites do not destroy other eggs in the host nest prior to laying their own, or when hosts can successfully fledge more than one parasitic nestling in the same brood (De Marsico, Mahler, & Rebores, 2010; Martinez, Soler, Soler, & Burke, 1998). Multiple parasitism may even be adaptive (from the parasite's perspective) if the presence of several parasitic eggs in the nest makes it more difficult for hosts to recognize them as foreign (Moskat et al., 2009; Payne, 1977; Stevens, Troscianko, & Spottiswoode, 2013) or to eject all of them (Peer, McCleery, & Jensen, 2018).

Laying decisions of female parasites may, in turn, influence selection on nestling virulence, since the relative frequencies of repeat and multiple parasitism affect the genetic relationships of parasitic nestlings within the same brood (Kilner, 2005; Rivers et al., 2012). Low genetic relatedness between parasitic nestmates should favour selfish behaviours, such as exaggerated begging and aggressive behaviours, whereas kinship among nestmates should mitigate selfishness. Genetic relatedness among parasites in the same nest could result from repeat parasitism by the same female or by multiple parasitism by related females and should favour reduced virulence by nestlings (Davies, 2000; Rivers & Peer, 2016).

The parasitic cowbirds (*Molothrus* spp.) vary in virulence across species and by host species. Although nestlings do not actively attack their foster siblings, female cowbirds typically puncture or remove other eggs that are already in the nest before laying their own (Fiorini et al., 2014, 2019; Peer, 2006). Nestlings may outcompete their nestmates for food, especially in nests of small-bodied hosts (Hoover, 2003; Lorenzana & Sealy, 1997; Tuero, Fiorini, & Rebores, 2007). In addition, some hosts will tolerate low levels of parasitism but abandon or reject heavily parasitized clutches (De Marsico, Gloag, Ursino, & Rebores, 2013; Peer et al., 2018). Assuming that these costs are sufficiently high – and that parasites can discriminate between host and parasitic eggs – females should avoid parasitizing nests that have already been parasitized by other females (experimentally demonstrated in brown-headed cowbirds, *Molothrus ater*; Ortega, Ortega, & Cruz, 1994). Repeat parasitism could be avoided if female cowbirds are capable of keeping a mental inventory of the locations of nests that they have already parasitized and avoid returning to them (the 'bookkeeping' hypothesis; Clayton, Rebores, & Kacelnik, 1997; Rebores, Clayton, & Kacelnik, 1996). Several studies have found behavioural and neuroanatomical evidence for these abilities in cowbirds, including sex differences in performance on spatial tasks (Guigueno, Snow, MacDougall-Shackleton, & Sherry, 2014) and hippocampus size (Guigueno, MacDougall-Shackleton, & Sherry, 2016; Rebores et al., 1996) that correlate with sex differences in nest-searching behaviours.

The hypothesis that female cowbirds should avoid repeat parasitism has received mixed support in the two species for which data are available, the brown-headed cowbird and the shiny cowbird, *Molothrus bonariensis*. In some populations, repeat parasitism is rare, apparently because females avoid returning to the same nest (Ellison et al., 2006; Gloag et al., 2014; Scardamaglia et al., 2017). In other populations, however, repeat parasitism has been observed and has been interpreted as a consequence of host scarcity (de la Colina, Hauber, Strausberger, Rebores, & Mahler, 2016; Ellison et al., 2006; McLaren et al., 2003; Rivers et al., 2012). This variation suggests that avoidance of repeat parasitism is somewhat flexible, depending on host availability as well as on the costs of intrabrood competition. However, systematic data on laying patterns are still lacking for the vast majority of brood parasites, largely due to the difficulty of matching parasitic eggs with the females who laid them and tracking the movements and laying behaviours of individual females (Feeney & Riehl, 2019).

In this study, we investigated patterns of repeat and multiple parasitism in the screaming cowbird, *Molothrus rufoaxillaris*, at a study site in Argentina. The screaming cowbird is the most specialized of the five *Molothrus* species, parasitizing only the greyish baywing, *Agelaioides badius* (hereafter 'baywing'), at this site and across most of its geographical range (Fiorini et al., 2019). The relationship between screaming cowbirds and greyish baywings is thought to reflect host–parasite coevolution, resulting in specialized parasitic adaptations including mimicry of nestling begging calls and nestling and fledgling plumages (De Marsico et al., 2012, 2019; Fraga, 1979; Ursino, Gloag, Rebores, & De Marsico, 2018). Despite this apparent coevolutionary history, egg laying by female screaming cowbirds is poorly synchronized with that of their hosts (approximately 50% of parasitic eggs are laid during the host's laying period; De Marsico & Rebores, 2008). Baywings reject parasitic eggs that are laid before their own first egg, but accept parasitic eggs laid after their own laying period has commenced. Hosts that are heavily parasitized may reject the entire clutch (including their own eggs) by using their feet to kick the eggs out of the nest cup and onto the rim of the nest, subsequently laying a second clutch in the same nest (De Marsico et al., 2012, 2013). Although parasitism by screaming cowbirds reduces baywing clutch size by approximately 22% (Cossa, Tuero, Rebores, & Fiorini, 2017; De Marsico & Rebores, 2014), hosts are typically able to fledge some of their own offspring along with one or more cowbird nestlings (De Marsico & Rebores, 2014).

Baywings at this site are heavily parasitized by screaming cowbirds: over 90% of host nests are typically parasitized with an average of approximately five cowbird eggs per host nest (De Marsico et al., 2013; De Marsico & Rebores, 2010). However, it is not known whether this results from repeated parasitism by the same female(s) or independent laying decisions made by multiple females. A recent study of radiotagged females revealed that individual home ranges overlap extensively in space and time, suggesting a lack of territorial behaviour (Scardamaglia et al., 2017; Scardamaglia & Rebores, 2014). Contrary to the predictions of the bookkeeping hypothesis, radiotagged females repeatedly visited nests that they had already parasitized; and in two of eight cases, a female laid a second egg in a nest that she had already parasitized (Scardamaglia et al., 2017). Given the relatively small number of nests and individuals monitored, however, it is not clear whether repeat parasitism is a common occurrence.

Here we use microsatellite genotyping of maternal DNA from screaming cowbird eggs to test the hypothesis that individual females avoid repeatedly parasitizing the same host nest. This hypothesis predicts that female cowbirds are more likely to lay successive eggs in different host nests than in the same nest, and that when multiple cowbird eggs are found in the same nest, these are laid by different rather than by the same female cowbird(s). To describe individual laying patterns, we also investigated whether eggs laid by the same female cowbirds were clustered in time and/or space across the breeding season and within the study area. Finally, in order to infer genetic relatedness of parasitic nestlings within broods, we also tested whether female cowbirds parasitizing the same host clutch were more or less closely related than chance would predict.

METHODS

Study Area and Field Sampling

Field work was conducted in Buenos Aires province, Argentina, at Reserva El Destino, a private 2400 ha reserve included in the Biosphere Reserve Parque Costero del Sur (MAB-UNESCO; 35°08'5.0316"S, 57°23'30.3072"W). It is primarily composed of wet

grassland habitat with patches of forest. Baywings are the main (almost exclusive) host of screaming cowbirds in this study site; both species are year-round residents. An alternative, infrequently used host, the brown-and-yellow marshbird, *Pseudoleistes virescens*, is present in the area but rarely nests at the study site. Baywings breed from early December to late February (Ursino, De Mársico, Sued, Farall, & Reboreda, 2011).

During the breeding season of 2013–2014, baywing nests were located, georeferenced and monitored every 1–3 days until young fledged or the nest failed (due to predation or abandonment). At each visit, we checked the nest contents and identified individual eggs to species using characteristics of background colour, spotting pattern and shape (Fraga, 1983, 1986; Friedmann, 1929). Parasitic eggs were collected before incubation began, or very early in incubation (<48 h), either from the nest cup itself or from the rim of the nest if hosts had already rejected them. To minimize behavioural effects of egg removal on the hosts, eggs taken from the nest cup were replaced with screaming cowbird eggs that had been previously collected as part of a different study, or with shiny cowbird eggs. These were removed when the hosts began incubation, or when the hosts naturally rejected them. An additional 26 cowbird eggs collected in the 2012–2013 breeding season were also included in the data set in order to ground-truth genetic assignments of egg maternity (see below).

During October–December 2012–2013, adult screaming cowbirds were captured using mist nets and cage traps baited with millet, banded with a unique combination of coloured and aluminium leg bands and genetically sampled via brachial venipuncture (20–30 μ l). Blood samples were stored at room temperature in lysis buffer (100 mM Tris, 100 mM EDTA, 10 mM NaCl, 2% SDS).

Genetic Analyses

We extracted maternal DNA from the membranes of unincubated eggs using Omega E.Z.N.A. Forensic Tissue Kits (D3396; Omega Bio-tek, Norcross, GA, U.S.A.) using the manufacturer's protocols with the following changes. First, eggs were incubated overnight at 56 °C in 1M EDTA, a chelating agent that decalcifies the shell and facilitates removal of the membrane (Igic et al., 2015). The entire membrane was then macerated with a scalpel and digested in 40 μ l of proteinase K overnight at 55 °C on a shaker. To ensure that genetic material from eggshell membranes represented maternal genotypes (from unincubated eggs) rather than embryonic genotypes (from eggs that had been partially incubated), we excluded any eggs that had visible embryonic development ($N = 16$; Strausberger & Ashley, 2001). We extracted DNA from adult females using Qiagen DNEasy Blood & Tissue kits (No. 69504; Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's protocols. All genetic samples were also sexed at diagnostic sex-linked alleles using the P2/P8 primer set (Griffiths, Double, Orr, & Dawson, 1998) and samples that appeared to be from males were excluded ($N = 12$).

We used a panel of 16 samples from adult screaming cowbirds to test 13 microsatellite loci that were initially developed for the brown-headed cowbird: CB1, CB12, CB15, Dp μ 15b, Dp μ 16, Ma μ 10, Ma μ 20, Ma μ 23, Ma μ 25, Ma μ 29, Ma μ 101, Ma μ 102 and Ma μ 104 (Alderson, Gibbs, & Sealy, 1999; Longmire et al., 2001; Strausberger & Ashley, 2001, 2003). Initial PCR conditions followed the original publications and the MgCl₂ concentration and annealing temperature were subsequently optimized for amplification. This preliminary analysis found eight of these loci to be polymorphic (Dp μ 16, Ma μ 25, Ma μ 29, Ma μ 101, Ma μ 102, CB1, CB12 and CB15). However, since other studies have found that the rate of PCR-based genotyping errors may be higher from DNA extracted from eggshell

membranes than from blood (due to the lower quantity and quality of genomic DNA from these samples; Riehl & Strong, 2019), we also genotyped a subset of 33 membrane samples in triplicate in order to estimate rates of genotyping error at each locus. Based on this analysis, CB12 showed evidence of stuttering (genotyping error rate ~12%) and Ma μ 102 showed a significant excess of homozygotes from expected Hardy–Weinberg proportions ($P = 0.0006$), so these two loci were excluded from analysis.

All samples were genotyped at the remaining six polymorphic microsatellite loci using a locus-specific forward primer fluorescently labelled at the 5' end (6-FAM, PET, NED or VIC; Applied Biosystems, Foster City, CA, U.S.A.). PCR products were sized on an ABI PRISM 3100 Genetic Analyzer with a GeneScan-500 LIZ molecular weight standard. Alleles were automatically scored using Geneious Prime (2019.2.1, Biomatters, Inc., San Diego, CA, U.S.A.) and visually checked by two observers (M.J.S. and C.R.). For all loci, alleles within one base pair of each other were binned together (i.e. alleles were required to differ by at least two base pairs). We used CERVUS 3.0 (Marshall, Slate, Kruuk, & Pemberton, 1998) to determine observed and expected heterozygosity levels, conformation to Hardy–Weinberg proportions and allele frequencies. This panel of loci had an overall genotyping error rate of 2.8%, did not deviate from Hardy–Weinberg equilibrium and gave a combined non-exclusion probability for identity of 6.0E⁻⁸. Summary statistics are given in Table 1.

Maternity Assignments

We used the 'identity analysis' function of CERVUS 3.0 to assign eggs in the study population to unique maternal genotypes (Marshall et al., 1998). For genotypes to be assigned to the same individual, we specified a genotyping error of 2.8% (calculated from technical replicates, above) and allowed mismatches at up to two loci, as long as the alleles differed by no more than two base pairs. We checked the accuracy of assignments in three ways. First, as a positive control, we included three eggs that were already known to have been laid by radiotagged females as part of a separate study (Scardamaglia et al., 2017). The identity analysis matched all three egg genotypes to the correct females. Second, as a negative control, we examined 14 pairs of eggs that were known to have been laid on the same day (and hence could not have been laid by the same female) and confirmed that the identity analysis excluded all 14 pairs as matches. Finally, we used the program COANCESTRY (Wang, 2011, 2017) to independently estimate genetic relatedness between all pairs of genotypes that CERVUS identified as matches, and confirmed that the 95% confidence intervals of the coefficients of relatedness included 1 (i.e. genetically identical; see details below).

Genetic Relatedness

We estimated genetic relatedness among different screaming cowbird females parasitizing the same baywing clutch and among all female genotypes in the population using the program COANCESTRY (Wang, 2011). Here we present analyses using the relatedness estimator of Queller and Goodnight (1989), but we ran the same analyses using three additional estimators (Lynch & Ritland, 1999; Ritland, 1996; Wang, 2017) and correlations between all four were high (>0.9). However, simulation analyses implemented in COANCESTRY using observed allele frequencies revealed that our panel of six microsatellite loci had wide confidence intervals around each estimate, indicating that we could not reliably identify second-order or more distant kin (Taylor, 2015). We therefore used bootstrapping (1000 bootstraps) to generate 95% confidence intervals around each estimate, and interpreted relatedness

Table 1
Characteristics of microsatellite loci used for identity analysis of a screaming cowbird population in Buenos Aires, Argentina

	<i>N</i>	<i>T_A</i> (°C)	<i>k</i>	PIC	<i>H_{obs}</i>	<i>H_{exp}</i>	NE-I	H–W (<i>P</i>)	Source
Dpu16	135	55	13	0.78	0.748	0.807	0.063	0.45	Alderson et al. (1999)
Mau101	116	55	11	0.66	0.629	0.702	0.126	0.65	Strausberger and Ashley (2001)
CB1	125	55	20	0.89	0.760	0.902	0.019	0.55	Longmire et al. (2001)
CB15	104	55	16	0.85	0.788	0.866	0.033	0.36	Longmire et al. (2001)
Mau25	119	55	7	0.43	0.504	0.485	0.322	0.96	Alderson et al. (1999)
Mau29	130	55	18	0.83	0.723	0.852	0.039	0.17	Alderson et al. (1999)

T_A = optimized annealing temperature; *k* = number of alleles per locus; PIC = polymorphic information content; *H_{obs}* = observed heterozygosity; *H_{exp}* = expected heterozygosity; NE-I = nonexclusion probability for identity; and H–W (*P*) = *P* value from chi-square test for deviation from Hardy–Weinberg equilibrium. Samples (*N* = 138) were taken in 2012–2014.

estimates very conservatively, as follows. Dyads were classified as ‘unrelated’ if the 95% confidence interval overlapped zero and the upper bound was below 0.25; ‘related’ if the lower bound of the 95% confidence interval was above 0.5; and ‘unknown’ if either the upper or lower bound of the 95% confidence interval fell between 0.25 and 0.5. To determine whether females parasitizing the same host nest were more or less closely related than chance would predict, we used bootstrapping (1000 bootstraps) to estimate the distribution of the mean difference in relatedness between dyads of females parasitizing the same host nest and between randomly chosen dyads in the population, and to determine whether the observed difference was statistically significant at $\alpha = 0.05$.

Sample Sizes

We monitored 57 greyish baywing nesting attempts (4 in 2012–2013 and 53 in 2013–2014) and recorded a total of 327 screaming cowbird eggs. Of these, 245 eggs were collected and 82 were not collected (most of these were eggs that were found after incubation had already begun at the host nest). Of the 245 eggs collected, the date of collection and location of the host nest were known for 220 eggs from 45 baywing clutches (this information was lost or not recorded for the remaining 25 eggs). An additional 28 eggs were excluded due to embryonic development. Of the remaining eggs, 114 were successfully genotyped (53%). Therefore, our final data set consisted of 101 genotyped eggs for which the location of the host nest and the collection date was known, and 13 genotyped eggs without that information. In addition, 24 adult females were trapped and genotyped, eight of which were assigned maternity to genotyped eggs. The 114 genotyped eggs were assigned to 78 unique maternal genotypes. Overall, therefore, we identified 94 unique female screaming cowbird genotypes in the study population (16 trapped adults that were not assigned to eggs, 8 trapped adults that were assigned to eggs, and 70 genotypes identified solely from eggs). For all analyses of laying by individual females, we restricted our data set to eggs collected in 2013–2014 because sampling in 2012–2013 was not sufficient to identify individual patterns.

Statistical Analyses

We used a binomial proportion test to determine whether female cowbirds were more likely to return to the same host nest or to parasitize a different host nest. To do this, we restricted the data set to females for whom we genotyped multiple eggs and knew the location of the host nest for each egg (*N* = 49 eggs laid by 18 females). Given the location of the first parasitic egg laid by each female, we then determined the number of instances in which each subsequent egg was laid in either a different host nest (0) or a previously parasitized host nest (1). The resulting observed proportion was compared to the null expectation of equal likelihood (50%) and 95% confidence intervals were used to estimate the two-

tailed probability that the true proportion differed significantly from the null.

To determine whether eggs laid by the same female were clustered temporally, we compared the distribution in laying intervals by each female (*N* = 49 eggs laid by 18 females) to the population-wide distribution of pairwise differences in lay date for all eggs in the study population (*N* = 98 eggs, 4753 pairwise comparisons). Bootstrapping (1000 bootstraps) was used to estimate the distribution of the mean difference in laying interval for individual females parasitizing the same host nest and for randomly chosen dyads of eggs in the study population, and to determine whether the observed difference was statistically significant at $\alpha = 0.05$. Similarly, to determine whether eggs laid by the same female were clustered spatially, we compared the distribution in pairwise distances between nests parasitized by the same female (*N* = 41 pairwise distances between nests parasitized by 15 females) to the population-wide distribution of pairwise distances for all parasitized nests in the study area (*N* = 1128 pairwise distances between 48 nests) and used the same bootstrapping approach to test for differences between the two distributions. We used the ‘point distance tool’ in ArcMap 10.7.1 (Environmental Systems Research Institute, ESRI, Redlands, CA, U.S.A.) to calculate pairwise distances. For females whose eggs were found in at least three different nests in the same breeding season (*N* = 6 females), we calculated home range sizes using minimum convex polygons in ArcMap 10.7.1.

Statistical tests were conducted in STATA 14 (StataCorp 2015, College Station, TX, U.S.A.). All tests were two tailed and results are given as means \pm standard errors unless otherwise noted.

Ethical Note

All work, including capture, handling, marking and blood sampling, complied with the ASAB/ABS Guidelines for the use of animals in research and with current laws in Argentina, and was performed under license from the Organismo Provincial de Desarrollo Sostenible, Argentina (Permit number 202/12-O.P.D.S.). Mist nets and cage traps were monitored continuously, and trapped individuals were removed immediately upon capture. During banding and blood sampling, adults and nestlings were held for under 10 min and the amount of blood taken was <1% of body mass. No mortality or other adverse effects were observed during capture or blood sampling, and no nests were abandoned as a result of monitoring or egg collection.

RESULTS

Timing of Parasitism

We recorded the stage at which the host nest was parasitized for 274 cowbird eggs. Of these, 35% were laid before the hosts began laying (*N* = 95), 29% were laid during the hosts’ laying period (*N* = 79) and 20% were laid during incubation (*N* = 54). The

remaining 16% were laid in re-nesting attempts after the hosts rejected the entire first clutch. Of these, 3% ($N = 9$), 7% ($N = 20$) and 6% ($N = 17$) were laid in the prelaying, laying and incubation periods, respectively. Overall, therefore, only 36% of cowbird eggs ($N = 99$) were laid synchronously with the host clutch. We recorded a total of 10 instances in which the hosts rejected the entire clutch, representing 43 cowbird eggs. Considering only nests for which we had data for the entire nesting cycle (prelaying through hatching), more parasitic eggs were laid during the prelaying stage than during laying or incubation. More parasitic eggs were laid asynchronously (relative to the host nesting cycle) than synchronously. Individual female screaming cowbirds had highly variable laying behaviour. Some females laid throughout the entire egg period (e.g. female genotype '8' parasitized clutches in the prelaying, laying and incubation periods), whereas others laid several eggs relatively synchronously with the host (e.g. female genotype '3' laid five eggs in the laying period and two eggs in the prelaying period).

Repeat and Multiple Parasitism

Baywing nests in the study area were heavily parasitized (55 of 57 nesting attempts, 96.5%). Most parasitized nests hosted more than one screaming cowbird egg (mean \pm SE = 5.7 ± 0.45 cowbird eggs per host nest; range 0–17; $N = 325$ eggs in 57 clutches). We collected an average of 5.10 ± 0.51 eggs per nest (range 0–13; $N = 245$ eggs in 48 clutches) and genotyped an average of 2.46 ± 0.18 eggs per nest (range 1–8; $N = 114$ eggs in 41 clutches). Multiple parasitism was very common (different parasite females laying in the same host clutch). Of 26 clutches for which we were able to genotype more than one cowbird egg, an average of 3.16 ± 0.35 different cowbirds laid eggs in the same clutch (range 2–8). Different cowbird eggs in the same host nest were typically laid by different females (Fig. 1). We also identified four cases in which the same parasitic female laid more than one egg in the same host nest. At three nests, two eggs were assigned to the same female; and in one nest, three eggs were assigned to the same female. However, of these four cases, only two represented repeat parasitism of the same nesting attempt. The other two occurred when the parasitic female laid one egg in a baywing clutch, then returned to lay another egg in the second nesting attempt after the baywings

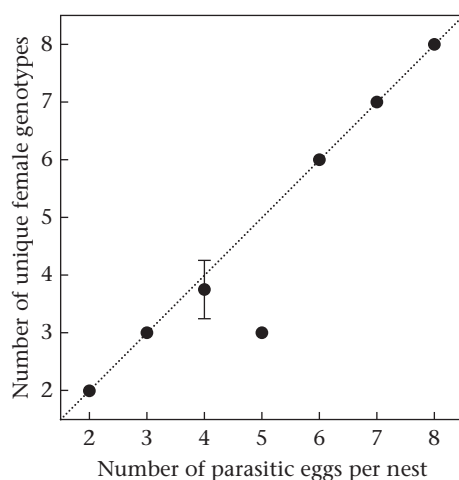


Figure 1. Number (mean \pm SE) of unique screaming cowbird genotypes in host nests that received two to eight parasitic eggs (two eggs, $N = 13$; three eggs, $N = 4$; four eggs, $N = 4$; five eggs, $N = 1$; six eggs, $N = 1$; seven eggs, $N = 1$; eight eggs, $N = 1$). The dotted line indicates the expected number of different genotypes if screaming cowbird females perfectly avoid repeat parasitism.

ejected the entire first clutch. Therefore, repeat parasitism of the same nesting attempt occurred in 2 of 57 clutches, 26 of which contained multiple cowbird eggs.

Individual female cowbirds were much more likely to lay in different host nests than they were to repeatedly parasitize the same nest (binomial proportion test: $z = 3.65$, $P = 0.0003$; $N = 49$ eggs laid by 18 females). For the 18 females that laid multiple genotyped eggs for which we knew the locations of the host nests, they laid an average of 2.8 ± 0.3 eggs per female (range 2–7) and parasitized an average of 2.5 ± 0.2 different host nests (range 1–4).

Overall, females parasitizing the same clutch did not differ significantly in relatedness from the population average (within-clutch mean $r \pm$ SE = 0.018 ± 0.026 ; $N = 145$ dyads; population-wide mean $r \pm$ SE = 0.034 ± 0.030 ; $N = 4560$ dyads; $P > 0.05$). We estimated coefficients of relatedness for 145 pairs of female genotypes collected from the same host nest. For 91 of these, the 95% confidence intervals were too wide for the dyad to be assigned to a relatedness category. Of the remaining 54 dyads, 48 consisted of genetically unrelated females and two dyads consisted of genetically related females.

Temporal and Spatial Patterns of Parasitism by Individual Females

Laying periods of female screaming cowbirds lasted up to 48 days (mean \pm SE = 20.0 ± 3.8 days, range 4–48 days; $N = 36$ eggs laid by 13 females). Some females laid throughout the entire study period (e.g. female genotype '1' laid 4 eggs over 37 days), whereas others laid several eggs in a short period (e.g. female genotype '2' laid 3 eggs in 5 days). The amount of time that elapsed between successive eggs was similarly variable (mean \pm SE = 12.0 ± 2.4 days; range 1–34 days; $N = 36$ eggs laid by 13 females). These figures should be interpreted as incomplete and descriptive, however, since genotyping was known to be incomplete and it is also possible that these female cowbirds laid eggs outside of our study area (see Sample Sizes). Even with incomplete sampling, however, eggs laid by the same females showed significant temporal clustering compared to a random distribution (mean inter-egg interval for individual female \pm SE = 12.0 ± 1.8 days; mean inter-egg interval for randomly chosen dyads of eggs \pm SE = 20.3 ± 0.3 days; Welch's t test: $t_{1433} = -4.4$, $P = 0.0002$).

Distances between nests parasitized by the same individuals were similarly variable (mean \pm SE = 1128.2 ± 124.6 m; range 14–3100 m; $N = 45$ pairwise distances between nests parasitized by 15 females). As with the time of laying, both lower and upper bounds of this estimate are truncated due to incomplete sampling. Despite this, eggs laid by the same females again showed significant spatial clustering compared to a random distribution (mean pairwise distance between all host nests in the study population \pm SE = 1541.2 ± 19.0 m; Welch's t test: $t_{2118} = 3.23$, $P = 0.002$). Female cowbirds had large home ranges that overlapped extensively. For the six females for whom we had sufficient data to calculate minimum convex polygon home ranges (≥ 3 nests parasitized), home range size ranged from 100.9 m^2 to 1.26 km^2 (mean \pm SE = $0.45 \pm 0.23 \text{ km}^2$). However, even the largest documented home range (1.26 km^2) was relatively small compared with the total area occupied by potential host nests in the study area (6.3 km^2).

DISCUSSION

Although multiple parasitism by screaming cowbirds is very common in this population of baywing hosts, genetic analyses revealed that this is primarily due to independent parasitism by unrelated females. Cowbird nestlings therefore regularly share baywing nests with unrelated conspecific nestmates, with up to

eight female cowbirds parasitizing a single brood. We found only four instances of repeat laying by the same female screaming cowbird in the same host nest, and two of these involved separate nesting attempts (i.e. different clutches) by the same host pair. Therefore, the overall rate of repeat parasitism in the same nesting attempt was approximately 7.7% (2 of 26 clutches). These results indicate that female cowbirds typically lay only one parasitic egg per host clutch, consistent with the bookkeeping hypothesis, even though baywings are capable of fledgling multiple parasitic nestlings.

This pattern is similar to that documented for generalist shiny, brown-headed and bronzed cowbirds, *Molothrus aeneus*, in which multiple parasitic eggs in the same host nest are typically the result of multiple parasitism by different females rather than repeat parasitism by the same female (de la Colina et al., 2016; Ellison et al., 2006; Gloag et al., 2014; but see Rivers et al., 2012). The rate of repeat parasitism by screaming cowbirds that we observed in this study (2 of 26 instances), although based on a larger data set, does not differ significantly from Scardamaglia et al.'s (2017) estimate from the same population, which found that two of eight instances represented repeat parasitism of the same nesting attempt (Fisher's exact test: $P = 0.27$). That study found that radiotagged screaming cowbirds at this site repeatedly visited host nests that they had already parasitized (between 2 and 39 visits in 1–9 days following parasitism), whereas shiny cowbird females rarely revisited the nests or nesting areas of any hosts that they had already parasitized (Scardamaglia et al., 2017). However, most visits by screaming cowbirds to previously parasitized nests did not result in laying, and were therefore interpreted as prospecting visits to determine whether the hosts had ejected the previously parasitized clutch.

The high frequency of multiple parasitism in this system raises the question of whether female cowbirds should actually benefit from avoidance of repeat parasitism, since searching for new host nests is time-consuming and potentially comes at the expense of foraging and other activities (Scardamaglia & Reboreda, 2014), and nestlings are likely to face competition from unrelated parasites anyway. One possibility is that a 'one egg per nest' rule is generally adaptive in populations with a low density of parasites relative to hosts. Avoidance of repeat parasitism could also be favoured if its main benefit is to spread the risk of failure among multiple host nests rather than to reduce intrabrood competition (i.e. a bet-hedging strategy; Andersson & Åhlund, 2012; Pöysä & Pesonen, 2007). Spreading risk among multiple nests might be similarly advantageous for screaming cowbirds, given their apparent inability to synchronize laying with their hosts.

Alternatively, several lines of evidence suggest that screaming cowbirds are unable to accurately assess the contents of host nests, which probably constrains their ability to synchronize laying with their hosts as well as their ability to avoid multiple parasitism. First, although screaming cowbirds frequently visit potential host nests, they rarely enter them (and are therefore unable to view the contents of the domed nests; Scardamaglia et al., 2017). Second, video recordings of parasitism events show that adult baywings often remain sitting on the nest cup when screaming cowbirds enter the nest, so the contents of the nest may not be visible to female parasites even when they are laying (De Mársico et al., 2013). Even when the nest contents are visible, the dark nest environment and visually similar eggs should make it difficult for cowbirds to discriminate between host eggs and those laid by other parasites (Fiorini et al., 2019). Given these constraints, female cowbirds have few opportunities to learn the appearance of their own eggs (or the appearance of host eggs), making learned discrimination of parasitic and host eggs very unlikely. The inability to visually inspect host nests for parasitic eggs may also favour the ability to

remember the location and stage of previously parasitized nests, since mental 'bookkeeping' may be the only way for females to avoid repeatedly parasitizing the same nest and accidentally damaging their own eggs (Sherry & Guigueno, 2019). In the two cases of repeat parasitism documented by Scardamaglia et al. (2017), one female laid one egg in the host's prelaying period and another egg during the host's laying period. The other female laid one egg during the host's laying period and another egg during the host's incubation period. Interestingly, in both cases of repeat parasitism that we documented in this study, all parasitic eggs were laid during the host's laying period (i.e. synchronously with the host). Moreover, in both cases, one of the host's eggs had been punctured, suggesting that in these instances the parasite was able to access the content of the nest and parasitize the nest twice at the appropriate stage in the host's laying cycle. It is therefore possible that repeat parasitism is most likely to occur at host nests that are likely to successfully hatch young. However, our data set was limited by the need to destructively sample cowbird eggs in order to determine maternal identity, as well as by a high rate of genotyping failure for collected eggs. Future studies on cowbird laying decisions and their fitness consequences, ideally involving both genetic and radiotracking data on individual females, are needed to rigorously test these hypotheses.

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