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RESEARCH ARTICLE

Kinship and genetic mating system of the Grayish Baywing (*Agelaioides badius*), a cooperatively breeding Neotropical blackbird

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ABSTRACT

Kin selection theory predicts that extrapair mating should be rare in cooperatively breeding birds. However, most cooperative breeders are not genetically monogamous and the relationship between promiscuity and cooperative breeding remains unclear. This relationship is further complicated by a lack of data. The majority of cooperatively breeding birds live in the tropics, and their genetic mating systems are little known. Here we studied the genetic mating system of the Grayish Baywing (*Agelaioides badius*), a socially monogamous Neotropical blackbird in which most nesting pairs are assisted by helpers, previously assumed to be offspring of the breeding pair. Grayish Baywings are the primary host of the parasitic Screaming Cowbird (*Molothrus rufoaxillaris*), and previous studies have found a positive association between brood parasitism and helper recruitment in the last part of the nestling period. We used microsatellite markers to analyze the kinship of 192 individuals in 47 breeding groups, finding that 13% of 153 nestlings (in 38% of 47 nests) resulted from extrapair mating. We also documented 2 instances of conspecific brood parasitism and 1 instance of quasiparasitism (the nestling was sired by the social father, but was unrelated to the social mother). Of 8 helpers that were genotyped, 4 (all males) were offspring of the breeding pair and 4 (2 males, 2 females) were unrelated to both members of the breeding pair. None of the helpers produced offspring within the clutch. These results suggest that, although cooperative breeding is frequent, genetic relatedness between Grayish Baywing helpers and the offspring that they raise is highly variable. Future studies are needed to determine why unrelated helpers assist at Grayish Baywing nests, and to understand the role that brood parasitism may have played in the evolution of cooperative breeding in this species.

Keywords: mating system, cooperative breeding, parentage analysis, extrapair mating, microsatellite markers, *Agelaioides badius*

Las relaciones de parentesco y el sistema de apareamiento genético del *Agelaioides badius*, un icterido neotropical con cría cooperativa

RESUMEN

La teoría de selección de parentesco predice que las cópulas por fuera de la pareja deberían ser raras en aves con cría cooperativa. Sin embargo, la mayoría de las aves cooperativas no son monógamas genéticas y la relación entre la promiscuidad y la cría cooperativa aún no es clara. Conocer esta relación es aún más complicado por la falta de datos: aunque la mayoría de las aves que se reproducen de forma cooperativa viven en los trópicos, sus sistemas de apareamiento genético son poco conocidos. En este trabajo, se estudió el sistema de apareamiento genético del *Agelaioides badius*, un ave neotropical que presenta monogamia social y en la que la mayoría de las parejas son asistidas por ayudantes, que fueron previamente asumidos como hijos de la pareja reproductiva. El *Agelaioides badius* es el principal hospedador del *Molothrus rufoaxillaris* y en estudios previos se ha encontrado una asociación positiva entre el parasitismo de cría y el reclutamiento de ayudantes en la última parte de la etapa de pichones. Se utilizaron marcadores moleculares para analizar 192 individuos de 47 grupos reproductivos, en los cuales se encontró que el 13% de 153 pichones (en 38% de 47 nidos) fueron resultado de cópulas por fuera de la pareja. También se documentaron dos eventos de parasitismo de cría conespecífico y un evento de cuasiparasitismo (el macho social fue el progenitor del pichón pero la hembra social no estuvo relacionada). De los 8 ayudantes que fueron genotipados, 4 (todos machos) fueron hijos de la pareja reproductiva y 4 (dos hembra y dos machos) no estuvieron relacionados con ambos miembros de la pareja reproductiva. Ninguno de los ayudantes produjo crías dentro de la nidada. Estos resultados sugieren que, a pesar de que la cría cooperativa es frecuente en esta especie, la relación genética entre los ayudantes y los pichones que ellos crían es muy variable. Son necesarios más estudios para determinar por qué ayudantes que no están emparentados asisten en los nidos de *Agelaioides badius* y para entender el rol que podría jugar el parasitismo de cría en la evolución de la cría cooperativa en esta especie.

Palabras clave: Sistema de apareamiento, cría cooperativa, análisis de parentesco, cópulas por fuera de la pareja, marcadores microsatélites, *Agelaioides badius*

INTRODUCTION

Cooperatively breeding birds live in social groups in which several individuals join together to rear a common clutch of young. In most cooperative breeders, a single reproductive pair is assisted by unpaired auxiliaries, or "helpers," who contribute to feeding and defense of the nestlings (Hatchwell 2009, Riehl 2013). Kin selection, in which individuals increase their inclusive fitness by helping family members, provides a powerful explanation for the evolution of cooperative breeding: Helpers are often previous offspring of the breeding pair, and are therefore closely related to the young that they help to raise (Hamilton 1964, Emlen 1995, Koenig and Dickinson 2004). Theory and recent comparative studies have suggested that helping behavior is more likely to evolve when females mate monogamously, since helpers and the young that they raise are full siblings ($r = 0.5$) if they share the same father and mother (Charnov 1981, Cornwallis et al. 2009). In contrast, helping behavior is predicted to be infrequent in populations with extrapair paternity, since promiscuous mating lowers genetic relatedness between helpers and the brood and diminishes the inclusive fitness benefits of cooperation (Cornwallis et al. 2009).

Empirical support for these predictions is mixed. Although a few cooperatively breeding birds appear to be truly genetically monogamous (e.g., the Florida Scrub-Jay [*Aphelocoma coerulescens*]; Townsend et al. 2011), the majority are not, and many are extraordinarily promiscuous. The Superb Fairywren (*Malurus cyaneus*), in which nearly 80% of nestlings may be fathered by males outside the social group, provides an infamous example (Mulder et al. 1994), and high rates of extrapair mating have been documented in a wide range of cooperatively breeding species (reviewed by Downing et al. 2015). In fact, Mulder et al. (1994) suggested that cooperative breeders might have *higher* rates of extrapair mating than their noncooperatively breeding counterparts, since the presence of helpers at the nest might enable females to seek extrapair matings even if this causes their social mates to lose paternity and reduce their investment in paternal care. Finally, a growing number of molecular studies have found that helpers are often entirely unrelated to the breeding pair (reviewed by Riehl 2013), raising the possibility that selective pressures other than kin selection may also be important drivers of cooperative breeding (Cockburn 1998, Clutton-Brock 2002, Kokko et al. 2002).

A comprehensive understanding of the relationship between genetic mating patterns, helper kinship, and cooperative breeding has been hampered by the biased geographic distribution of available data: Although the majority of cooperatively breeding species live in the tropics (Cockburn 2006), studies of the reproductive biology of temperate-zone birds outnumber those of their tropical counterparts by more than 100 to 1 (Macedo et al. 2008, Stutchbury and Morton 2008). To our knowledge, molecular information on extrapair paternity is available for only a small minority of Neotropical species with helpers at the nest: the Bicolored Wren (*Campylorhynchus griseus*; Haydock et al. 1996); White-throated Magpie-Jay (*Calocitta formosa*; Berg 2005); Greater Ani (*Crotophaga major*; Riehl 2012); Campo Flicker (*Colaptes campestris*; Dias et al. 2013); and White-banded Tanager (*Neothraupis fasciata*; Moreira 2014).

In this study, we used microsatellite analysis to determine the genetic relationships between breeding adults, helpers, and nestlings of the Grayish Baywing (*Agelaioides badius*), a cooperatively breeding Neotropical blackbird. Breeding pairs are socially monogamous, and both males and females participate in nest defense and parental care (Fraga 1991, Ursino et al. 2011). Up to 95% of pairs are assisted by 1–4 unpaired helpers, which typically join the group after the nestlings have hatched and assist in nest defense and food delivery (Fraga 1991, Ursino et al. 2011). The number of helpers may increase over the nestling and fledgling stages, such that a breeding group can consist of up to 10 adults and the brood (Fraga 1991). A previous observational study of color-banded nestlings found that some helpers were older offspring of the breeding pair who had remained on the natal territory rather than dispersing, whereas others were breeding adults from nearby territories whose nests had failed (possibly also relatives of the breeding pair; Fraga 1991). Grayish Baywings are sexually monomorphic, and neither the sex of the helpers nor their kin relationships have been previously determined.

In our study population in Argentina, Grayish Baywing nests are heavily parasitized by the host-specialist Screaming Cowbird (*Molothrus rufoaxillaris*; 93–100% of nests are parasitized annually, with an average of 5.0 ± 0.3 eggs per parasitized nest) and occasionally parasitized by the host-generalist Shiny Cowbird (*Molothrus bonariensis*; 16% of nests parasitized annually, with an average of 1.4 ± 0.1 eggs per parasitized nest; De Mársico et al. 2010). Cowbird nestlings do not directly attack Grayish Baywing nestlings; rather, they are slightly larger than host nestlings, beg more aggressively, and can outcompete them for food (Lichtenstein 2001, De Mársico and Reboreda 2010, 2014). High rates of brood parasitism may have implications for the evolution of cooperative breeding, as helpers increase the rate of food delivery to the nest and may increase the probability that the breeding pair can successfully raise their own nestlings along with any parasitic nestlings (Ursino et al. 2011). Interestingly, nests that were parasitized by Shiny and Screaming cowbirds recruited more helpers during the posthatching period than unparasitized nests, suggesting that the decision to help is partly influenced by the level of provisioning required by the brood (Ursino et al. 2011). Therefore, helpers may be able to substantially increase offspring fitness in this system, accruing some indirect fitness benefits even if genetic relatedness is low.

Our objectives in this study were to understand (1) the genetic mating system of the Grayish Baywing, including rates of extrapair mating and conspecific brood parasitism; (2) the kin relationships between nestlings, the breeding pair, and their helpers; and (3) the sex of the helpers. We evaluated the hypothesis that cooperative breeding in Grayish Baywings is driven primarily by the indirect fitness benefits of aiding kin by testing the predictions that (1) rates of extrapair mating are low, and (2) Grayish Baywing helpers are genetically related to the brood that they help to raise.

METHODS

Study Area and Field Sampling

Fieldwork was conducted in the province of Buenos Aires, Argentina, at Reserva El Destino, a private 2,400-ha reserve that was part of the Biosphere Reserve Parque Costero del Sur (MAB-UNESCO; 35.13°S, 57.38°W). The reserve consisted of wet grassland habitat interspersed with patches of forest, primarily *Celtis ehrenbergiana* and *Scutia buxifolia* (Cagnoni et al. 1996). Grayish Baywings were year-round residents and bred from late November to late February. From 2006 to 2014, Grayish Baywing nestlings were banded at 8 days of age with a unique combination of colored bands and a numbered aluminum band. From 2011 to 2012, adult Grayish Baywings were captured during the nonbreeding season (September–October) using cage traps baited with millet. During the breeding season, unbanded adults were subsequently captured at active nests using mist nets after the eggs had hatched (Ursino 2016). As with nestlings, adults were banded with a unique combination of colored bands and a numbered aluminum band. For both adults and nestlings, 20–30 μL blood samples were taken by brachial venipuncture and stored at room temperature in lysis buffer (100mM Tris, 100 mM EDTA, 10 mM NaCl, 2% SDS). Banding and bleeding were not observed to have any adverse effects on the survival of nests or nestlings.

Grayish Baywings rarely build their own nests, instead using secondary cavities, nest boxes, or nests previously built by other species (primarily the enclosed nests of furnariids, including *Phacellodomus* spp., *Synallaxis* spp., and *Furnarius rufus*; and tyrannids, including *Pitangus sulphuratus*; Fraga 1988, De Mársico et al. 2010). Approximately 10–25% of Grayish Baywing nests monitored annually were built in wooden nest boxes, which had been present in the study area since 2003 (detailed in De Mársico et al. 2010). Grayish Baywings do not renest after a successful breeding attempt (Fraga 1991, De Mársico and Reboreda 2008). Fewer than 40% of Grayish Baywing nests produce fledglings in a given reproductive season (De Mársico and Reboreda 2008, 2010). Between 60% and 77% of nests fail shortly after laying due to desertion or nest predation, and ~14% of nests are depredated during the nestling stage (De Mársico and Reboreda 2008).

During the breeding seasons of 2006–2007 and 2009–2014, Grayish Baywing nests were located and monitored every 1–2 days until young fledged or the nest failed (due to predation or abandonment). The contents of the nest, the number of adults present, and the identity of marked individuals were recorded during each visit. We defined an individual as a helper when we observed a new banded or unbanded individual with a banded pair at the nest; helpers were generally easy to distinguish from the breeding pair because they did not occur at nests until relatively late in the nesting cycle (after nest building, incubation, and laying). Permanent markers were used to number eggs and to identify nestlings for recognition within the clutch. Nestlings were uniquely marked with waterproof ink on one leg, both legs, or neither leg.

The eggs and nestlings of the Screaming Cowbird appear similar to those of the Grayish Baywing. Parasitic eggs were identified using the characteristics proposed by Fraga (1986), including background color patterning and shape. Nestlings younger than 5 days of age were identified by bill and skin color (following Fraga 1979); after 5 days of age, however, identification was visually impossible due to high host–parasite similarity (De Mársico 2009). In contrast, Shiny Cowbird eggs were easily distinguished by shape, background color, and patterning, and nestlings by the coloration of skin, bill, and emerging primary feathers (De Mársico 2009).

Sample Sizes

It was difficult to obtain genetic information for all eggs within Grayish Baywing clutches, as female Screaming Cowbirds often puncture host eggs when parasitizing the nest, some eggs fail to hatch, and some are depredated

(De Mársico and Reboreda 2010, 2014). For our analysis, we used nestling genotypes from a total of 47 clutches, primarily from complete clutches ($n = 27$) or clutches in which only 1 egg was lost ($n = 19$). For 6 of 27 complete clutches, we were unable to confirm the original clutch size because the nest was found after laying had been completed; we defined these as complete clutches if no predation occurred during the rest of the monitoring period. For 2 clutches, samples were obtained from just 2 nestlings of 4 eggs laid; these were included in the analysis because we were able to obtain blood samples from at least 1 social parent. Within these 47 breeding groups, we genotyped a total of 192 blood samples from 153 nestlings, 8 helpers, and 31 social parents (18 females and 13 males). All 8 helpers were unpaired and joined the nesting pair after nestlings had hatched ($n = 6$ groups). However, this represented only a minority of the total number of helpers in the population, most of which we could not capture. Blood samples were obtained from all nestlings and both social parents ($n = 9$ clutches), 2 nestlings and both social parents ($n = 1$ clutch), 2 nestlings and 1 social parent ($n = 1$ clutch), all nestlings and 1 social parent ($n = 10$ clutches), and all nestlings and neither social parent ($n = 26$ clutches). Samples were collected between 2006 and 2014 as follows: 2006–2007, $n = 7$ clutches; 2009–2010, $n = 7$ clutches; 2010–2011, $n = 2$ clutches; 2011–2012, $n = 10$ clutches; 2012–2013, $n = 7$ clutches; and 2013–2014, $n = 14$ clutches (for more details see Appendix Table 3).

Molecular Sexing and Genotyping

Genomic DNA was extracted from whole blood samples using OMEGA E.Z.N.A. Tissue DNA kits (D3396; Omega Bio-tek, Norcross, Georgia, USA) following the manufacturer's protocols. Adults associated with nests (social parents and helpers) were sexed via polymerase chain reaction (PCR) amplification of the CHD-Z and CHD-W alleles using the P2/P8 primer pair (Griffiths et al. 1998) in combination with the P0 primer (Han et al. 2009).

We used a panel of 16 samples from adult Grayish Baywings trapped across the study area to test 13 microsatellite loci that were initially developed for the Brown-headed Cowbird (*Molothrus ater*): CB 1, CB 12, CB 15, Dpμ 15b, Dpμ 16, Maμ 10, Maμ 20, Maμ 23, Maμ 25, Maμ 29, Maμ 101, Maμ 102, and Maμ 104 (Alderson et al. 1999, Longmire et al. 2001, Strausberger and Ashley 2001, 2003). Initial PCR conditions followed the original publications and the $MgCl_2$ concentration and annealing temperature were subsequently optimized for amplification. Of the 13 previously published loci, 6 were not used in our analysis: Maμ 23 and Maμ 25 did not successfully amplify for most individuals; Maμ 20, Maμ 101, and Maμ 102 were not polymorphic (<3 alleles); and Dpμ 15b presented evidence of stuttering. The remaining 7 microsatellite loci that were used in our analysis were highly polymorphic and showed no evidence of genotyping errors or linkage disequilibrium (Table 1).

TABLE 1. Characteristics of microsatellite loci used for parentage analysis of a Grayish Baywing population in Buenos Aires, Argentina. Samples were taken in 2006–2007 and 2013–2014. T_A = optimized annealing temperature; k = number of alleles per locus; H_o = observed heterozygosity; H_e = expected heterozygosity; and H-W = P-value from test for deviation from Hardy-Weinberg equilibrium.

Locus	T_A (°C)	k	H_o	H_e	H-W
CB 1	55	14	0.750	0.783	0.22
CB 12	55	20	0.888	0.882	0.71
CB 15	55	24	0.881	0.906	0.11
Dpμ 16	55	18	0.630	0.655	0.18
Maμ 10	63	10	0.508	0.487	0.54
Maμ 29	55	19	0.881	0.877	0.66
Maμ 104	55	14	0.702	0.734	0.19

All samples were genotyped for the 7 polymorphic microsatellite loci using a combination of the fluorescently labeled universal primer M13 and modified locus-specific primers with 5′ universal primer sequence tails. Up to 4 loci were multiplexed in the same PCR reaction using a locus-specific forward primer fluorescently labeled at the 5′ end (6-FAM, PET, NED, or VIC; Applied Biosystems, Foster City, California, USA). PCR products were sized on an ABI PRISM 3100 Genetic Analyzer with a GeneScan-500 LIZ molecular weight standard and GeneMapper 3.0 software (Applied Biosystems). We used the free program Micro-checker 2.2.3 (Van Oosterhout et al. 2004) to determine levels of genotyping error, and we used Cervus 3.0 (Marshall et al. 1998) and GENEPOP 4.2 (Jennings and Blanchard 2004, Rousset 2008) to determine observed and expected heterozygosity levels, conformation to Hardy-Weinberg proportions, null allele frequencies, and gametic disequilibrium between locus pairs. To quantify the discrimination power of this set of 7 microsatellite loci, we calculated the probability of identity (P_{ID}), which is the probability that 2 individuals in a population have identical genotypes. The estimated P_{ID} was very low (2.95×10^{-9} for randomly chosen individuals in the population, 1.3×10^{-3} for full siblings), indicating high resolution (Waits et al. 2001).

Assignment of Kinship

We assigned kinship between (1) social parents and offspring (paternity and maternity); (2) nestlings within the same clutch; and (3) helpers and the breeding pair and their nestlings.

For clutches in which we genotyped at least one social parent, we calculated the probability that the adult was the genetic parent of each nestling in the clutch. For clutches in which we genotyped a helper, we also calculated the probability that the helper was the genetic parent of each of the nestlings in the clutch, and the probability that the helper was the offspring of each member of the breeding pair. For each candidate parent and offspring, we used Cervus 3.0 to calculate the natural logarithm of the likelihood ratio (LOD score), which estimates the probability that the candidate parent is the genetic parent of the offspring relative to a randomly chosen individual from the study population. We used simulations from allele frequency data in the population to calculate the critical differences in LOD scores (ΔLOD) between the most likely and second most likely candidate parent that were necessary for assignment with 95% confidence. Simulations were run for 100,000 cycles and the estimated genotyping error was 1%.

We next analyzed genetic relationships between nestlings in the same clutch for breeding groups for which we possessed genotypes for nestlings only ($n = 26$), or nestlings and only 1 parent ($n = 11$). We used the maximum likelihood approach implemented in Kingroup 2.0 (Konovalov et al. 2004) to estimate 5 different measures of the coefficient of relatedness (r) between each pair of nest-mates within a clutch (defined by Goodnight and Queller 1999, Lynch and Ritland 1999, Smith et al. 2002, Wang 2004, Konovalov and Heg 2008). Full siblings should theoretically exhibit $r = 0.50$, half siblings $r = 0.25$, and unrelated individuals $r = 0.00$. However, as estimates of r are continuous and lack discrete boundaries between unrelated, half, and full siblings, we first estimated the coefficients of relatedness between known full siblings (from nests at which both parents were genotyped; $n = 25$ individuals and 24 dyads from 9 nests). Ninety-nine percent confidence intervals for each r estimate were calculated by bootstrapping, and values of r consistent with a full sibling relationship (FS) were defined as falling within the lower and upper bounds of this range. We then generated a similar dataset for r estimates of unrelated nestlings (UR) by randomly selecting dyads ($n = 48$) of unrelated nestlings ($n = 24$) from different nests across the study area, and calculating 99% confidence intervals. Unfortunately, sample sizes for known half siblings (HS) were insufficient to estimate the range of r values ($n = 2$). Therefore, r values for half-sibling relationships were conservatively defined as those that fell between the upper limit of the range for known unrelated nestlings and the lower limit of the range for known full siblings. Of the 5 estimates of r tested, that of Konovalov and Heg (2008) was the most conservative and resulted in the fewest incorrect assignments, so we present this estimator for all analyses.

Finally, we verified all kinship assignments by directly comparing the genotypes of social parents, nestlings, and helpers (Woxvold and Mulder 2008). Adults were excluded as genetic parents if their genotypes were incompatible with the nestling's at >1 locus (i.e. if neither allele was shared between the adult and the nestling at ≥ 2 loci). Similarly, nestlings within the same clutch were excluded as full siblings if >4 alleles were present at >1 locus (i.e. if there was evidence at ≥ 2 loci that >2 adults contributed to the clutch; Woxvold and Mulder 2008). Genotypes of helpers were compared with those of the breeding pair and their nestlings in the same way. Direct comparison of genotypes is more conservative than parentage assignments based on allele frequencies, and was therefore considered to provide an estimated lower bound to the rate of extrapair mating in this population. Inconsistencies at a single locus were conservatively considered to be the result of genotyping error.

When a half-sibling relationship was detected within a clutch and/or the social father of a clutch was excluded as the genetic father of a nestling within the clutch, this was considered to be the result of extrapair paternity. When a half-sibling relationship was detected and the social father was assigned as the genetic father of the nestling, but the social mother was excluded as the genetic mother of the nestling, this was considered to be the result of quasiparasitism. Finally, if both social parents were excluded as genetic parents of the nestling, it was considered to be the result of conspecific brood parasitism.

RESULTS

Genetic Mating System

Of the 31 social parents (18 females and 13 males) that were genotyped from 21 nests, all but 4 (13%) were assigned as the genetic parents of all of the nestlings in the nest with $\geq$95% confidence. Two of the 4 remaining cases were consistent with extrapair paternity: (1) the social mother was assigned as the genetic mother for all nestlings, but the social father was assigned as the genetic father for 2 of 3 nestlings, and (2) the social mother was assigned as the genetic mother for 2 nestlings, but the social father was excluded as the genetic father of the same nestlings. One case was consistent with quasiparasitism: the social father was assigned as the genetic father for 1 nestling in the clutch, but the social mother was excluded as the genetic mother. Finally, the fourth case was consistent with either conspecific brood parasitism or with quasiparasitism: the social mother was excluded as the genetic mother for 1 of 2 nestlings in the clutch, but we could not obtain the genotype of the social father. We subsequently used Kingroup 2.0 to calculate relatedness between the 2 nestlings in this clutch and found that they were unrelated ($r = -0.92$), consistent with conspecific brood parasitism. Therefore, of the 60 nestlings in 21 nests

TABLE 2. Two estimates of the numbers and frequencies of full siblings (FS), half siblings (HS), and unrelated nest-mates (UR) of Grayish Baywings in Argentina, 2006–2007 and 2013–2014, derived from kinship assignment programs (left) and direct comparison of genotypes (right). Cervus 3.0 (Marshall et al. 1998) was used for parentage assignment of nestlings for which both parental genotypes were known, and Cervus 3.0 was used in combination with relatedness estimates derived from Kingroup 2.0 (Konovalov et al. 2004) when the genotypes of one or both social parents were unknown.

		Kinship assigment		Direct comparison	
No. of social parents genotyped	Relationship	No. nestlings	Percent nestlings	No. nestlings	Percent nestlings
Both social parents	FS	24	86	24	86
	HS	4	14	4	14
	UR	0	0	0	0
Subtotal		28	100	28	100
One social parent	FS	28	88	30	94
	HS	3	9	1	3
	UR	1	3	1	3
Subtotal		32	100	32	100
Neither social parent	FS	78	84	85	91
	HS	14	15	7	8
	UR	1	1	1	1
Subtotal		93	100	93	100
All categories	FS	130	85	139	91
	HS	21	14	12	8
	UR	2	1	2	1
TOTAL		153	100	153	100

for which we genotyped at least 1 social parent, 2 nestlings in 2 nests were not genetic offspring of the social mother (quasiparasitism or conspecific brood parasitism) and 3 nestlings in 2 nests were not genetic offspring of the social father (extrapair paternity). Direct comparison of genotypes was consistent with the assignments made with Cervus 3.0 in all cases (Table 2).

We then used Kingroup 2.0 to reanalyze the genetic relationships between nest-mates for the 11 groups for which we obtained genotypes for only 1 social parent, as well as for the clutches for which we lacked parental genotypes. For the former dataset, relatedness estimates for 3 nestlings indicated that they were half-siblings of the other nestlings in the clutch ($0.08 < r < 0.21$; Table 2), suggesting cases of extrapair mating that were not detected in the first analysis due to incomplete sampling. For the dataset lacking parental genotypes, 14 of 93 nestlings (15%) in 14 of 26 clutches (54%) shared coefficients of relatedness with nest-mates that indicated extrapair mating. In these cases, we were not able to distinguish between extrapair paternity and quasiparasitism. Finally, 1 additional case of conspecific brood parasitism was detected. As expected, estimates of extrapair mating by direct comparison of nestling genotypes were more conservative than those calculated by Kingroup 2.0 (Table 2).

Combining the parentage results obtained using Cervus 3.0 and nest-mate relationships obtained with Kingroup 2.0, we found evidence of extrapair mating in 20 of 153 nestlings (13%) from 18 of 47 nests (38%; Table 2, Figure 1). Only 1 case of quasiparasitism and 2 cases of conspecific brood parasitism were confirmed in the 47 reproductive groups (Table 2, Figure 1).

Kinship of Helpers

We genotyped 8 unpaired helpers belonging to 6 breeding groups and used Cervus 3.0 to estimate their relatedness to the breeding pair and the nestlings in the clutch. Four helpers (2 females and 2 males) were not assigned as

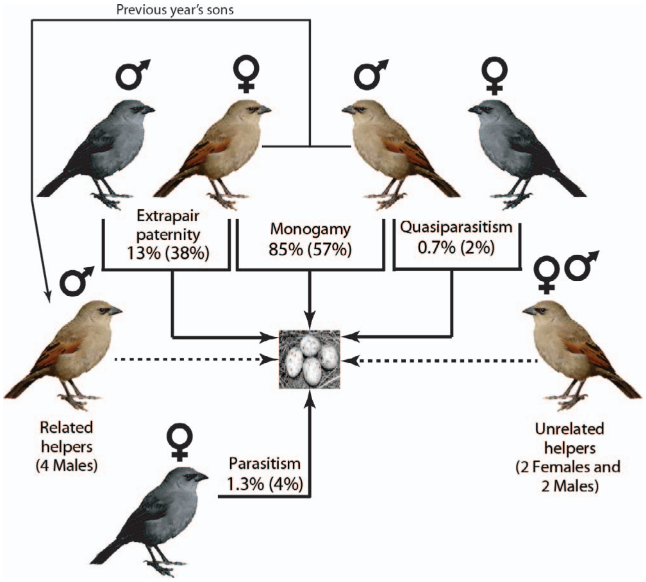

FIGURE 1. Percentage of Grayish Baywing nestlings ($n = 153$) produced within the pair, by extrapair paternity, by quasiparasitism, and by conspecific brood parasitism in Argentina, between the 2006–2007 and 2013–2014 reproductive seasons (November–February). The percentage of nests ($n = 47$) is given in parentheses. Individuals in the social group are shown in brown and those outside the social group are shaded in gray.

offspring of the reproductive pair or as genetic parents of any nestling in the clutch (Figure 2). However, in 1 case we did not obtain genetic information on the social father of the breeding group; therefore, we used Kingroup 2.0 to estimate Konovalov's coefficients of relatedness between the helper, the social mother, and the nestlings of this group. All pairwise relationships indicated that the helper was unrelated to the rest of this breeding group ($-0.47 < r < 0.03$). The 4 unrelated helpers were all first observed at the nest when the nestlings were between 3 and 9 days of age.

The other 4 helpers (all males) were first observed at the nest when the nestlings were between 2 and 4 days old. All of them were assigned as genetic offspring of the breeding female (the social mother) at the nest. We were able to obtain genetic information on the social father in only 1 case, and it was assigned as the genetic father of the helper at the 95% confidence level by Cervus 3.0. In the other 3 cases, coefficients of relatedness between the helper and the nestlings indicated that the helpers were full siblings of the nestlings ($0.36 < r < 0.80$; Figure 2). These results were consistent with direct comparisons of the genotypes, and suggested that these 4 helpers were genetic offspring of the breeding pair.

Extrapair paternity occurred at only 1 nest with helpers. Of the 2 helpers at this nest, 1 was a related male (a son of the breeding pair) and 1 was an unrelated female, but neither helper was assigned as a genetic parent of the nestlings.

DISCUSSION

Our results suggest that cooperatively breeding Grayish Baywings are socially monogamous with frequent extrapair copulations: 13% of nestlings in 38% of nests resulted from extrapair mating, and a single case of quasiparasitism and 2 cases of intraspecific parasitism were also detected. Compared with other socially monogamous passerines, this rate of infidelity is moderate (Griffiths et al. 1998) and is lower than that of many cooperatively breeding birds (Sachs and Rubenstein 2007, Cornwallis et al. 2009). Of the 4 helpers in this study that were previous offspring of the breeding pair, all were males and were full siblings of at least some of the nestlings that they attended. However, the other 4 helpers were genetically unrelated to either the breeding pair or to the brood. This suggests that social groups can form either through delayed dispersal of offspring (primarily males), or through recruitment of unrelated individuals (either males or females).

In some cooperatively breeding birds, unrelated males appear to gain direct fitness benefits by helping at a pair's nest; for example, by copulating with the breeding female or by inheriting the mate and territory if the breeding male dies (Reyer 1986; reviewed by Riehl 2013). In an earlier study of Grayish Baywing mating behavior, Fraga (1991) observed 4 instances in which the breeding female copulated with unpaired helpers at the nest, and speculated that unrelated helpers might attempt to sire young in the clutch. However, all 4 of these copulations occurred after egg-laying had already ceased, so none resulted in the production of young. Similarly, we found that helpers did not reproduce in the brood that they were currently assisting. One possibility is that unrelated males practice a "best-of-a-bad-job" strategy by helping at nests (for example, after their own reproductive attempts have failed) in return for a low probability of producing offspring. Fraga (1991) observed 1 case of apparent cooperative polyandry in which 2 male Grayish Baywings copulated with the same female and provided paternal care at her nest, suggesting that male helpers might reproduce if they are able to join a breeding pair while the female is still fertile. Alternatively, unrelated helpers might help at nests in order to increase their chances of inheriting a mate or territory in future reproductive attempts (Piper et al. 1995), of recruiting future help from the nestlings that they help to rear, or of recruiting reciprocal help from the breeding pair in future nesting attempts. However, we lack direct evidence in support of any of these alternative hypotheses.

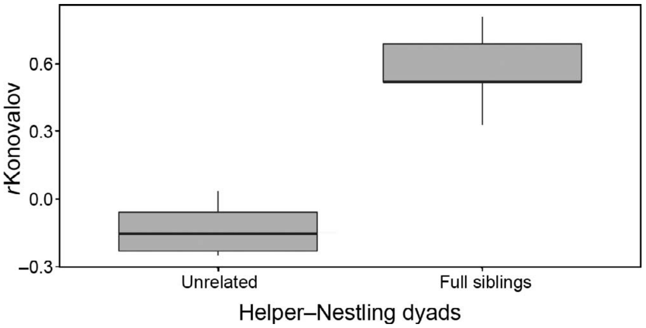

FIGURE 2. Coefficients of relatedness (*r*Konovalov; Konovalov's coefficient of relatedness estimated using Kingroup 2.0; Konovalov et al. 2004) for helper–nestling dyads (verified from pedigree analysis with parental genotypes) of Grayish Baywings in Argentina between the 2006–2007 and 2013–2014 reproductive seasons (November–February). Boxes show median and interquartile range; whiskers show minimum and maximum.

The high frequency of brood parasitism in this population may also affect the fitness costs and benefits of helping. Brood parasitism might negatively affect the inclusive fitness of related helpers, both by reducing the number of related offspring in the brood (since female Screaming Cowbirds typically puncture one host egg and replace it with their own), and by increasing the amount of provisioning effort expended on unrelated parasitic nestlings (Ursino et al. 2011). However, if the additional food provided by helpers significantly increases the chances of host nestlings fledging from parasitized clutches, then helping at parasitized nests could substantially increase the indirect fitness of helpers. All 3 recorded host species of the Screaming Cowbird are cooperative breeders (Di Giacomo and Reboreda 2015), suggesting that cowbirds may preferentially parasitize cooperative breeders because they provide better parental care—or, conversely, that parasitism may favor cooperation if helpers can mitigate the negative effects of cowbird nestlings on host nestling survival (Feeney et al. 2013).

In summary, our study revealed variable mating patterns in cooperatively breeding Grayish Baywings, as well as frequent helping (but no reproduction) by nonrelatives. Longer-term studies with larger sample sizes are now needed to determine whether unrelated helpers reap future direct fitness benefits, how the presence of helpers influences the long-term reproductive success of breeders, and how brood parasitism interacts with parental care to influence the costs and benefits of cooperative behavior.

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Ethics statement: This study was conducted under license from the local authority on protected areas (Provincial Organism for Sustainable Development, Buenos Aires, Argentina) and complied with the current laws of Argentina. No animals were harmed during manipulations, and nest abandonment following banding and bleeding was not observed (we continued to monitor all Grayish Baywing nests until chicks fledged or the nest was depredated).

Author contributions: C.A.U. and M.C.D.M. carried out the fieldwork and collected the samples; C.A.U. carried out the lab work and data analysis; C.R. supervised the lab work; J.C.R. supervised the field research and general project system; and C.A.U. and C.R. wrote the paper. All authors contributed substantially to conceiving ideas, formulating hypotheses, and interpreting the data.

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APPENDIX TABLE 3. Clutch characteristics, numbers of nestlings and adults genotyped, and results of genetic analyses for all Grayish Baywing (*Agelaioides badius*) nests used in this study in Argentina, 2006–2014. For the number of helpers, "Present" refers to the number of helpers observed at the nest during the nestling stage and "Genotyped" refers to the number of helpers that were captured and sampled. For the proportion of the brood resulting from extrapair copulations (EPCs), "Cervus" indicates estimates derived from Cervus 3.0 (Marshall et al. 1998), "*r*" indicates estimates derived from Kingroup 2.0 (Konovalov et al. 2004), and "Direct" indicates estimates derived from direct observations of genotypes. "MD" denotes missing data.

Nest	Year	Initial clutch size	No. Baywing eggs lost to parasites	No. parasitic eggs	No. nestlings genotyped	No. social parents genotyped	Number of helpers		Proportion of brood resulting from EPCs		
							Present	Genotyped	Cervus	*r*	Direct
1	2006–2007	5	0	0	5	0	MD	0		0.20	0.20
2	2006–2007	4	0	3	4	0	1	0		0.25	0.25
3	2006–2007	MD	MD	MD	3	0	2	0		0.00	0.00
4	2006–2007	4	0	5	4	0	0	0		0.00	0.00
5	2006–2007	4	0	8	4	0	1	0		0.00	0.00
6	2006–2007	4	0	1	4	0	MD	0		0.00	0.00
7	2006–2007	4	0	2	4	0	1	0		0.25	0.25
8	2009–2010	5	0	3	5	0	0	0		0.25	0.00
9	2009–2010	4	0	3	4	0	0	0		0.25	0.25
10	2009–2010	3	0	3	3	0	0	0		0.25	0.00
11	2009–2010	4	0	3	4	0	0	0		0.00	0.00
12	2009–2010	3	0	0	3	0	1	0		0.33	0.00
13	2009–2010	4	1	7	3	0	0	0		0.33	0.00
14	2009–2010	3	1	9	3	0	0	0		0.33	0.00
15	2010–2011	3	0	16	3	0	0	0		0.00	0.00
16	2010–2011	3	0	5	3	0	2	0		0.00	0.00
17	2011–2012	4	0	6	3	1	1	1	0.00	0.00	0.00
18	2011–2012	5	1	11	4	0	0	0		0.00	0.00
19	2011–2012	MD	MD	MD	3	2	0	0	0.00		0.00
20	2011–2012	4	2	8	2	2	0	0	0.00		0.00
21	2011–2012	MD	MD	MD	4	1	0	0	0.00	0.00	0.00
22	2011–2012	3	1	7	2	2	0	0		0.00	0.00
23	2011–2012	3	0	7	3	0	1	0		0.33	0.33
24	2011–2012	4	0	5	3	2	0	0		0.00	0.00
25	2011–2012	4	1	9	3	2	1	0	0.00	0.00	0.00
26	2011–2012	4	0	3	3	1	0	0	0.00	0.00	0.00
27	2012–2013	4	0	6	3	2	0	0	0.66		0.66
28	2012–2013	4	1	2	3	2	1	1	0.00		0.00
29	2012–2013	3	1	3	2	1	0	0	0.00	0.00	0.00
30	2012–2013	4	0	3	3	2	2	2	0.00		0.00
31	2012–2013	4	1	3	3	1	2	0	0.00	0.00	0.00
32	2012–2013	5	1	1	4	1	1	0	0.00	0.00	0.00
33	2012–2013	MD	MD	MD	3	1	1	0	0.00	0.00	0.00
34	2013–2014	4	0	2	4	0	0	0		0.25	0.25
35	2013–2014	4	0	5	3	2	1	1	0.00		0.00
36	2013–2014	4	1	5	3	1	1	1	0.00	0.33	0.33
37	2013–2014	3	0	3	3	2	1	0	0.00		0.00
38	2013–2014	4	1	1	3	0	0	0		0.33	0.33
39	2013–2014	3	0	4	3	0	0	0		0.33	0.00
40	2013–2014	3	0	6	2	1	0	0	0.50	0.50	0.50
41	2013–2014	MD	MD	MD	3	0	0	0		0.00	0.00
42	2013–2014	MD	MD	MD	3	0	0	0		0.00	0.00
43	2013–2014	4	1	5	3	0	1	0		0.00	0.00
44	2013–2014	4	1	7	2	1	0	0		0.00	0.00
45	2013–2014	4	1	3	3	0	0	0		0.00	0.00
46	2013–2014	4	0	3	4	0	0	0	0.00	0.25	0.25
47	2013–2014	4	0	8	4	1	2	2	0.00		0.00
TOTAL					153	31	24	8			