

Family ties in a neotropical cooperative breeder: within-group relatedness and fine-scale genetic structure in the greyish Baywing (*Agelaioides badius*)

JUAN MANUEL ROJAS RIPARI,^{1*} LEONARDO CAMPAGNA,^{2,3} BETTINA MAHLER,¹ IRBY LOVETTE,^{2,3}
JUAN CARLOS REBOREDA¹ & MARÍA CECILIA DE MÁRSICO¹

¹Departamento de Ecología, Genética y Evolución & IEGEBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Ciudad Universitaria, Buenos Aires, C1428EGA, Argentina

²Fuller Evolutionary Biology Program, Cornell Lab of Ornithology, Ithaca, NY, USA

³Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA

Cooperative breeding is a reproductive system in which one or more adults (helpers) assist others in rearing their offspring. Cooperative breeding occurs in 9% of birds, encompassing remarkable variation in mating systems and patterns of social organization. Understanding how this diversity evolved requires assessing social and genetic relationships across a broad range of cooperative systems. Yet, for some geographical regions like the Neotropics, detailed genetic studies of cooperatively breeding birds are still comparatively scarce. We used double-digest restriction-site associated DNA sequencing to define the genetic relationships within and between cooperative groups in a Neotropical facultative cooperative breeder, the Greyish Baywing *Agelaioides badius*. Between 2015 and 2018, we banded and sampled adults and nestlings of 41 breeding groups from a population in the Province of Buenos Aires, Argentina. We found that helpers were primarily males, the majority of which were genetically related to the individuals they assisted, either as previous offspring of the breeding pair or as first- or second-order relatives of the breeding male. However, we also observed unrelated male and female helpers. Parentage analysis indicates that reproduction was strongly skewed towards the dominant pair and helpers never gained paternity within and only once outside their social group. This scenario is consistent with helping behaviour being driven primarily by kin selection rather than by direct genetic benefits to helpers. However, the occasional occurrence of helpers unrelated to the breeding pair suggests that non-reproductive direct benefits such as increased access to resources or future breeding opportunities may also influence helping decisions in this species. We found weak evidence consistent with male natal philopatry, and fine-scale genetic structure, as adult males in our population showed higher relatedness at close geographical distances than females. Future studies that further examine the mechanisms behind group formation and the fitness pay-offs of helping behaviour will increase our understanding of the complex cooperative system of the Greyish Baywing.

Keywords: double-digest restriction-site associated DNA sequencing, kin selection, Neotropics, social organization.

Cooperative breeding is a reproductive system in which one or more adults (helpers) provide

parental care to young that are not their own (Stacey & Koenig 1990). This mode of parental care is known to occur in approximately 9% of bird species, with particularly high prevalence in Australia and sub-Saharan Africa (Ligon & Burt 2004, Cockburn 2006, Feeney *et al.* 2013,

*Corresponding author.

Email: jmrojasripari@ege.fcen.uba.ar

Twitter: @jmrojasripari

Wells & Barker 2017). Helpers-at-the-nest have posed a long-standing evolutionary paradox because they invest time and energy in raising other birds' offspring at the expense of delaying or forgoing their own independent reproduction. One explanation for this apparently altruistic behaviour has been provided by kin selection theory, which poses that helping others can be favoured if the genetic benefits to the helper outweigh the fitness costs associated with this behaviour (Hamilton 1964, Griffin & West 2003). Consistent with this theory, cooperative breeding in vertebrates is more commonly directed to kin, so helpers can increase their inclusive fitness by enhancing the reproductive success of the related individuals that they assist (Cockburn 2006, Hatchwell 2009, Riehl 2013, Hatchwell *et al.* 2014, McDonald 2014, Warrington *et al.* 2014, Riehl & Stern 2015, Leedale *et al.* 2020).

However, over the past decades, the extended use of molecular tools to determine genetic relationships has revealed a more complex picture than previously thought. Although less frequent than kin-directed cooperation, helping also occurs between non-kin, and it may involve a combination of related and unrelated helpers within the same cooperative group (Seddon *et al.* 2005, von Lippke 2008, Riehl 2011, 2013). Cooperative breeding is associated with a wide variety of mating systems, from mainly monogamous (Ekman & Ericson 2006, Hatchwell 2009, Cramer *et al.* 2011) to highly promiscuous (Cockburn 1998, Richardson *et al.* 2001, Hughes *et al.* 2003, Riehl 2013). This diversity has renewed questions about the importance of inclusive vs. direct fitness benefits as drivers of helping behaviour, especially when kin-selected benefits to helpers are not evident and there is conflict between breeders and helpers over reproduction and parental care (Cockburn 2013, Riehl 2013, Cockburn *et al.* 2016, Shen *et al.* 2016, Kaiser *et al.* 2019). A comprehensive understanding of the evolution of helping behaviour can only be achieved by assessing social and genetic patterns across a broad range of cooperative systems. Yet, for some geographical regions like the Neotropics, detailed genetic studies of cooperatively breeding birds are still comparatively scarce, despite being the most biodiverse region of the world (Cockburn 2006, Riehl 2011, Dias *et al.* 2013, Brouwer & Griffith 2019, Santos & Macedo 2019). Quantitative estimates of kinship using genetic data are available for only 14 species

(nine from South America) out of 111 Neotropical bird species for which there is evidence of cooperative breeding (Cockburn 2006, Riehl 2013, Wells & Barker 2017).

Determining kinship and parentage within social groups is a necessary first step to characterizing social organization and breeding systems. However, to better understand the role of kinship in promoting helping behaviour, fine-scale population structure should also be considered (Hatchwell 2010). Genotypes of structured populations are not randomly distributed in space and genetic differentiation tends to increase with geographical distance (Wright 1943). At fine geographical scales, genetic structure can shape behavioural traits such as territoriality (Lee *et al.* 2010) and kin-based cooperation. The latter trait has often arisen in populations showing spatial association of kin, mainly as a result of the retention of philopatric offspring (Woxvold *et al.* 2006, Hatchwell 2010, Ribeiro *et al.* 2012). The spatial association of kin can also be passively achieved by short dispersal distances. In birds, philopatry is typically biased towards males, whereas females more often disperse and breed at greater distances from their natal territory (Clarke *et al.* 1997, Double *et al.* 2005, Woxvold *et al.* 2006, Ribeiro *et al.* 2012). However, the reverse pattern has also been observed (Woxvold *et al.* 2006, Berg *et al.* 2009, Ribeiro *et al.* 2012), as well as the absence of sex-biased dispersal (Blackmore *et al.* 2011). Dispersal patterns may have important implications for the expected fitness pay-offs of helping behaviour for each sex, thus ultimately influencing the social organization and genetic structure of animal societies (Richardson *et al.* 2002, Dickinson & Hatchwell 2004, Ribeiro *et al.* 2012, Green & Hatchwell 2018). Such sex-biased dispersal patterns can be studied by searching for correlations between geographical and genetic distances (e.g. through spatial autocorrelation analysis), which can differ for each sex.

Here, we examine genetic relationships within and between social groups in a Neotropical cooperative breeder, the Greyish Baywing *Agelaioides badius* (hereafter Baywings), a medium-sized passerine native to southern South America. Baywings are socially monogamous and facultative cooperative breeders (Fraga 1991, Ursino *et al.* 2011). Breeding pairs can have one to four helpers that join almost exclusively during the nestling and fledgling stages. Previous studies

suggest that helpers are predominantly males and may be young adults that did not disperse after fledging, although there is also evidence of adult individuals redirecting help after failing with their own breeding attempt (Fraga 1991, Ursino *et al.* 2017). However, the main patterns of relatedness within and between social groups are not well understood. Based on banding data, Fraga (1991) reported nine out of 12 helpers marked as nestlings that assisted either one or both parents, but also observed an unrelated helper. Ursino *et al.* (2017) assessed within-group relatedness for eight helpers using a panel of seven microsatellites and showed that half of them (all males) were the previous offspring of the assisted pair, and the other half (two males and two females) were unrelated to both breeders. This study also revealed extra-pair paternity at 38% of 47 sampled nests and occasional intraspecific brood parasitism (i.e. nestlings genetically unrelated to both breeders) and quasi-parasitism (i.e. nestlings that were sired by the breeding male but unrelated to the breeding female). The high prevalence of extra-pair nestlings suggests helping may be associated with direct benefits for both male and female helpers, including the access to extra-pair mating opportunities (e.g. Kaiser *et al.* 2019). However, the extent of reproductive sharing within cooperative groups has not been reliably established for Baywings, and it is not clear yet whether helpers gain reproductive benefits within or outside their groups.

Our aim in this study was to expand on previous work and assess the potential for kin-selected helping behaviour in Baywings by examining social organization and spatial genetic patterns. We take advantage of the high resolution provided by reduced-representation genomic techniques to infer genetic relationships within social groups as well as to assess fine-scale genetic structure in the population. We hypothesized that social groups are formed through the delayed or limited dispersal of males, as this is the most typical pattern in birds. Our predictions, if philopatry is male-biased, are that genetic relatedness between adult males is negatively correlated with the geographical distance between their respective social groups, and that males exhibit, on average, higher between-group relatedness with same-sex neighbours than adult females. Regarding the role of kin selection, if helping in Baywings is driven primarily by indirect genetic benefits, we expect helpers to be close

relatives of at least one member of the breeding pair they assisted, and not to gain parentage within or outside their cooperative group.

METHODS

Study population

We studied a population of Baywings near the town of Magdalena in the Province of Buenos Aires, Argentina (35°08'S, 57°25'W). The study location is a flat area (c. 10 m above sea level) of approximately 300 ha, located in the private reserve El Destino (c. 2400 ha), within the Biosphere Reserve Parque Costero del Sur (MAB-UNESCO). It comprises remnants of native semi-deciduous forest, locally known as *talares* dominated by *Celtis ehrenbergiana* and *Scutia buxifolia*, with a lower abundance of *Jodina rhombifolia* and *Schinus longifolius* (Cagnoni *et al.* 1996). The forest patches are embedded within a matrix of tall grasslands (*Stipa* sp.) and marshes (*Spartina* sp. and *Juncus* sp., Pagano & Mérida 2009). Baywings are year-round residents in the area, where they breed from late November to mid-February. They rarely build their own nests, but use closed or domed nests of other species, including those of furnariids (Firewood-gatherer *Anumbius annumbi*, Thornbirds *Phacellodomus* spp., Spinetails *Synallaxis* spp., *Furnarius rufus*) and tyrannids (*Pitangus sulphuratus*), secondary cavities like woodpecker (*Colaptes* spp.) nests and nestboxes (De Mársico *et al.* 2010).

Nest monitoring and capture

We conducted the study during three breeding seasons (2015/16, 2016/17 and 2017/18), and searched exhaustively for Baywings' nesting attempts throughout each season. All nests found were georeferenced with a GPS (Garmin Etrex10) and checked every 1–3 days until young fledged or the nest failed. Baywings are the main host of the brood-parasitic Screaming Cowbird *Molothrus rufoaxillaris* and almost all nests are parasitized (De Mársico *et al.* 2010). We marked all eggs individually using Sharpie® markers and assigned them to Greyish Baywing or Screaming Cowbird based on differences in background colour and spotting pattern (Fraga 1983). Hatchlings of each species were identified using the colour of the skin and bill tip as diagnostic cues

(Fraga 1979), and uniquely marked on their tarsi with permanent ink. From the late incubation period onwards, we attempted to capture all attending adults using mist-nets placed near the nesting tree. We marked every captured adult uniquely with a combination of coloured plastic bands and a numbered metal ring, and took a small blood sample (c. 30–50 μ L) by brachial venepuncture. Breeders and helpers could be differentiated because the latter joined the nest after hatching. Banding of Baywings in the study area started in 2008 (Ursino *et al.* 2017), so we also kept records of previously banded individuals recruited to the monitored nests. In subsequent visits to the nests, we recorded the identity of the adults present to corroborate group membership. Briefly, we conducted daily focal observations and video recorded every nest during the nestling stage (see Rojas Ripari 2020 for additional details). Because of this level of monitoring we are confident that we identified all of the visiting adults in each social group. Baywing nestlings were banded and blood was sampled in the same way as for adults on day 8 post-hatching. By that age, Baywing nestlings have a mass of about 30 g, which allowed us to extract a similar blood volume to that taken from adults (Owen 2011). Some adults that were not associated with a breeding group were alternatively captured with walk-in traps baited with millet. The location of capture was georeferenced to be used in spatial genetic analyses.

DNA extraction and sex determination

Blood was collected in heparinized capillary tubes and stored immediately in 0.5 mL of lysis buffer (100 mM Tris-HCl, 100 mM ethylenediamine tetraacetic acid, 10 mM NaCl, 2% sodium dodecyl sulphate) at room temperature until DNA extraction at the end of each field season. We extracted DNA from blood samples of 75 adults and 97 nestlings following an isopropanol-NaCl procedure modified from an ethanol-NaCl protocol (Miller *et al.* 1988). We also included in our analysis an additional set of blood samples from 28 individuals that were colour-banded before 2015 (Ursino *et al.* 2017) and re-sighted as breeders or helpers during the present study period. DNA from these 28 samples was extracted using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Although our blood samples were stored in lysis buffer at room

temperature (which can lead to DNA degradation over longer periods of time; reviewed by Owen 2011), this was not an issue in our study because DNA was extracted shortly after the field season ended and did not show signs of degradation (e.g. high levels of missing data, see SNP filtering section below). Adult samples were sexed by polymerase chain reaction (PCR) amplification of the sex-linked CHD gene using primers 2550F/2718R (Fridolfsson & Ellegren 1999, Lee *et al.* 2010). The PCR technique produced visible clear bands on agarose gels (see Supplementary material, Fig. S1) and assays were repeated in case 'ghost' bands appeared in order to avoid false male assignments. Breeding females that were sexed in the field with morphological (brooding patch) and behavioural cues (only females brood) did not contradict the PCR results.

Double-digest restriction site-associated DNA protocol and sequencing

Double-digest restriction site-associated DNA sequencing (ddRAD-seq) and bioinformatic processing for single nucleotide polymorphism (SNP) discovery and genotyping was adapted from Peterson *et al.* (2012) following Thrasher *et al.* (2018). The DNA concentration of sample extracts was determined using the Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Samples were then diluted or concentrated to 5–25 ng/ μ L to obtain a final mass of 100–500 ng of DNA in a volume of 20 μ L. Briefly, the samples were digested with the restriction enzymes *Sbf*I-HF (NEB, Ipswich, MA, USA; R3642L) and *Msp*I (NEB; R0106), and the resulting fragments were ligated to P1 (with a unique barcode) and P2 (with an index group barcode) adapters to the 5' and 3' ends, respectively. We pooled all samples, each containing a unique combination of barcodes, for posterior bioinformatic demultiplexing. The DNA libraries were size-selected by BluePippin (Sage Science, Beverly, MA, USA) to retain fragments between 400 and 700 bp and amplified using nine PCR cycles with Phusion DNA Polymerase (NEB). Following clean-up with AMPure beads, index groups were pooled in equimolar ratios and sequenced on one lane of an Illumina HiSeq 2500 at the Cornell University Biotechnology Resource Center to obtain single-end 101-bp reads.

Bioinformatic processing and *de novo* assembly of RAD loci

The sequence reads obtained were subjected to a quality filtering process with the *FastQC* 0.11.5 program (www.bioinformatics.babraham.ac.uk/projects/fastqc). We trimmed all sequences to 97 bp using *fastX_trimmer* (FastX-Toolkit) to exclude low-quality base calls near the 3' end of the reads. Additionally, reads containing one or more bases with a Phred quality score below 10 or with more than 5% of the positions below 20 were discarded using the tool *fast_quality_filter* (FastX-Toolkit). The sequences were then demultiplexed using the *process_radtags* module from the STACKS pipeline (Catchen *et al.* 2013) to obtain sequences for each individual. Given that a sequenced genome of the Greyish Baywing (or a close relative) is not available, we assembled the filtered sequences *de novo* into RAD loci using the *denovo_map.pl* script from the STACKS pipeline (Catchen *et al.* 2013, Rochette & Catchen 2017), setting a minimum stack depth of 10 (parameter *m*), a maximum of four mismatches per locus within an individual (*M* parameter) and a maximum of four differences between alleles in the population (*n* parameter). When reference genomes of the focal or closely related species are available, a reference-based assembly of RAD loci is preferred (Shafer *et al.* 2017, Thrasher *et al.* 2018, Lois *et al.* 2020). However, we opted for a *de novo* assembly instead of a reference-based assembly using the genome of a more distantly related species, because in the latter case the percentage of mapped reads tends to be low (Peterson *et al.* 2012, Campagna *et al.* 2015).

Single nucleotide polymorphism filtering

We used the populations module of STACKS to generate an SNP dataset, retaining loci present in at least 80% of the individuals (*r* parameter). We restricted our data set to only one SNP per locus (*-write_single_snp*) to avoid including tightly linked markers, and set the minor allele frequency to 0.1 (*min_maf*). Null alleles cannot be automatically filtered (Flanagan & Jones 2019), but we mitigated this potential problem with our choice of higher values for the *m* and *min_maf* parameters, with our Hardy–Weinberg equilibrium filter, and with the settings used in the posterior relatedness estimator (Andrews *et al.* 2016, see [Relatedness](#)

[analysis](#) section below). The depth of coverage, averaged across loci, ranged from 24.94× to 70.86× reads per sample. Finally, loci that were not in Hardy–Weinberg equilibrium were removed using VCF tools v0.1.16 (Danecek *et al.* 2011). The resulting dataset (327 SNPs, 10% of missing data on average) was exported in variant call format (*.vcf*), converted to Genepop (Rousset 2008) and formatted to be suitable for analysis using *related* (Pew *et al.* 2015, see SOM Appendix S1) using PGDSpider v2.1.1.5 (Excoffier & Lischer 2010) and CERVUS 3.0.7 (Kalinowski *et al.* 2006). We conducted a power analysis in CERVUS version 3.0.7 and found that the mean expected heterozygosity ($H_{\text{exp}} = 0.35$) was comparable to that of another SNP dataset obtained for the same purposes as ours ($H_{\text{exp}} = 0.45$ in Thrasher *et al.* 2018), but lower than in the previous microsatellite-based study in our focal species ($H_{\text{exp}} = 0.76$ in Ursino *et al.* 2017). This difference is due to the biallelic nature of our SNP markers. However, the probability of identity (P_{ID}), which is the probability of two individuals in the same population sharing the same genotype, was much lower than in the microsatellite-based study ($P_{\text{ID}} = 1.95 \times 10^{-102}$ for randomly chosen individuals and $P_{\text{ID}} = 1.8 \times 10^{-52}$ for full siblings compared with $P_{\text{ID}} = 2.95 \times 10^{-8}$ and $P_{\text{ID}} = 1.3 \times 10^{-3}$, respectively, in Ursino *et al.* 2017).

Relatedness analyses

Relatedness between individuals was assessed using the package *related* (Pew *et al.* 2015) in R version 4.0.3 (R Core Team 2020). This package accounts for genotyping errors and missing data, and can estimate relatedness using various non-likelihood-based and likelihood-based estimators. To obtain best performance, we compared different estimators (using the command *compareestimators*) and chose the most suitable one by simulating pairs of individuals with the allele frequencies observed in our adult sub-sample for each of these kinship categories: half siblings, full siblings, parent–offspring and unrelated. The simulated data allowed us to plot the expected values for each kinship category by building density maps. We chose the triadic likelihood estimator of Wang (2007, Fig. S2) which provided maximum resolution with minimum overlap between each kinship category. We estimated pairwise between *breeding pair and helper(s)* (Appendix S3), between *helpers and*

assisted nestlings (Appendix S4) and between the breeding pair and raised nestlings (Appendix S5). The first two sets of estimates served to determine the putative role of adults within cooperative groups (i.e. breeders or helpers) when not all adults could be sampled for genetic analyses. To complement the above results, we estimated the kinship categories (half siblings, full siblings, parent–offspring and unrelated) using the software ML-Relate (Kalinowski *et al.* 2006).

Fine-scale genetic structure

We searched for fine-scale genetic structure and analysed sexual dispersion patterns in our study population by conducting a spatial autocorrelation analysis (Smouse & Peakall 1999). We used the software GenAlEx (Peakall & Smouse 2012) to correlate the genetic distance between adult Baywings with geographical distance between nests or the GPS point of capture (UTM coordinates) for adults that were not associated with a nest (Appendix S2). The calculated genetic distance is not strictly a measure of kinship/relatedness (Loiselle *et al.* 1995, Ritland 1996) or relationship (Cockerham 1969), but rather reflects pairwise genetic similarity. These estimators are correlated and using genetic distances allows us to pool data from the three seasons (Smouse & Peakall 1999, Smouse *et al.* 2008). We estimated spatial autocorrelation for adults of both sexes, and for the males and females separately, considering evenly spaced distance classes (i.e. 0–100 m, 100–200 m, etc.). We defined the shortest distance class based on the minimum dispersal distance recorded for banded juveniles in our study population that were re-sighted as breeding adults in the first year after fledging (*c.* 100 m, pers. observ.). Autocorrelation analyses excluded helpers to avoid spurious results regarding the association of relatives at the shortest spatial scale. We estimated the overall significance of each correlogram by integrating all correlation estimates (r) using a heterogeneity test (ω). The ω estimator accounts for the overall significance of the whole correlogram and enables us to compare patterns between sexes in the same population. Given that this test involves bootstrapping resampling and permutations, we set a more conservative α level of 0.01 to consider the contrasted correlograms significant (Banks & Peakall 2012). We also analysed spatial autocorrelation considering increasing distance classes (i.e. 0–100 m,

0–200 m, etc.), using the Multiple Dclass option in GenAlEx (Peakall & Smouse 2006).

RESULTS

Helper sex-ratio is skewed to males

We successfully sequenced and genotyped individuals from a total of 41 breeding groups across the three reproductive seasons (see Supplementary material, Table S1), 20 of which were cooperative groups (41% in 2015, 53% in 2016, 64% in 2017) with 1.26 helpers on average (range: 1–3). Most of the analysed helpers were of unknown origin, because they were captured as adults upon joining the breeding pair after the eggs hatched. In two cooperative groups, we genotyped either the social male or the breeding pair but could not sample the observed helpers (Table S1). Twenty-one of the 23 sampled helpers were males, indicating a strong sex bias in helping behaviour. One of the two genotyped female helpers was observed simultaneously assisting two neighbouring nests.

Both related and unrelated helpers occur at Baywing nests

The ML-Relate analysis assigned male helpers as previous offspring of the breeding pair ($n = 5$ helpers at two nests), as first- or second-order relatives of the breeding male ($n = 8$ helpers at eight nests), or as unrelated to both breeders ($n = 7$ helpers at six nests). The female helpers were both unrelated to the breeding pairs they assisted (Fig. 1a,b). Another helper that could not be genotyped because of a limited number of sequencing reads (although sex was successfully determined using a PCR-test) was assigned as previous offspring of the breeding pair through banding records. It was banded as a nestling in 2015 and observed assisting its (at least social) parents the following season (marked with an asterisk in Fig. 2b).

We analysed the relatedness of helpers to the brood they assisted at 19 nests from which genotypes of nestlings, helpers and at least one breeder were obtained. The individual mentioned above that was not genotyped was considered to be a full sibling of the nestlings raised in 2016 as for the majority of nests present genetic monogamy of chicks with respect to their parents was recovered (62% and 67% in Ursino *et al.* 2017 and this

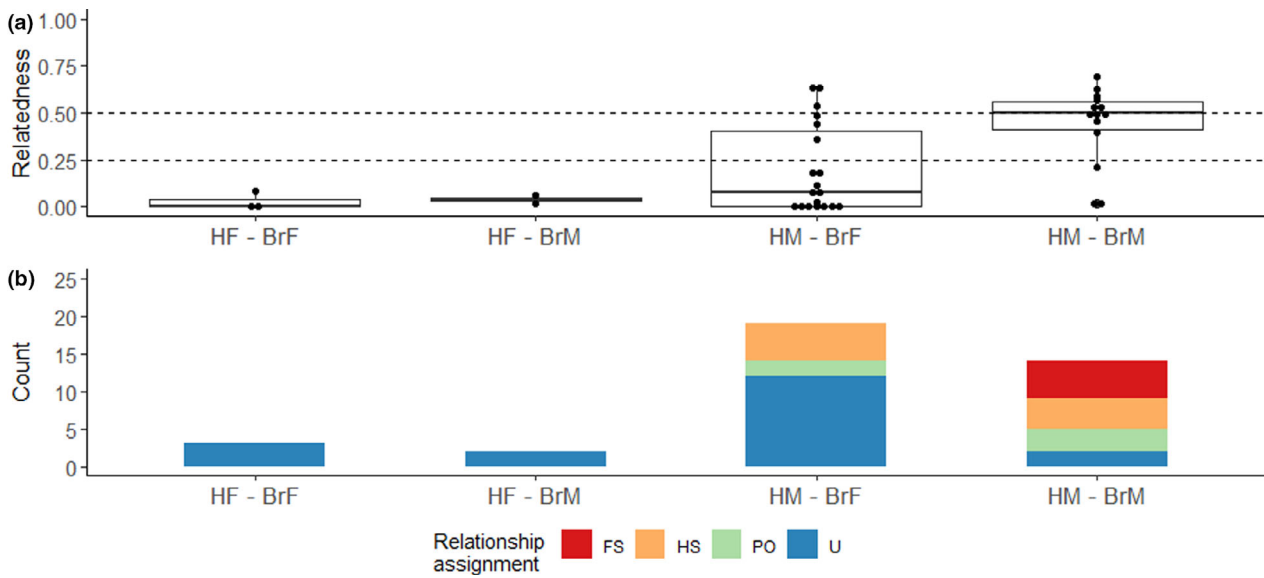


Figure 1. (a) Average relatedness (mean ± se) of male helpers (HM, $n = 21$) and female helpers (HF, $n = 2$ where one helper assisted two broods simultaneously) to the breeding female (BrF) and male (BrM) they assisted. Dotted lines indicate the expected relatedness coefficients for first-order ($r = 0.5$) and second-order ($r = 0.25$) relatives. Boxes indicate the mean relatedness value as a bold line, its standard error in the box contour and whiskers are 95% confidence values. (b) Relationships assigned by ML-Relate between helpers and assisted breeders (FS: full-sibling, HS: half-sibling, PO: parent-offspring, U: unrelated).

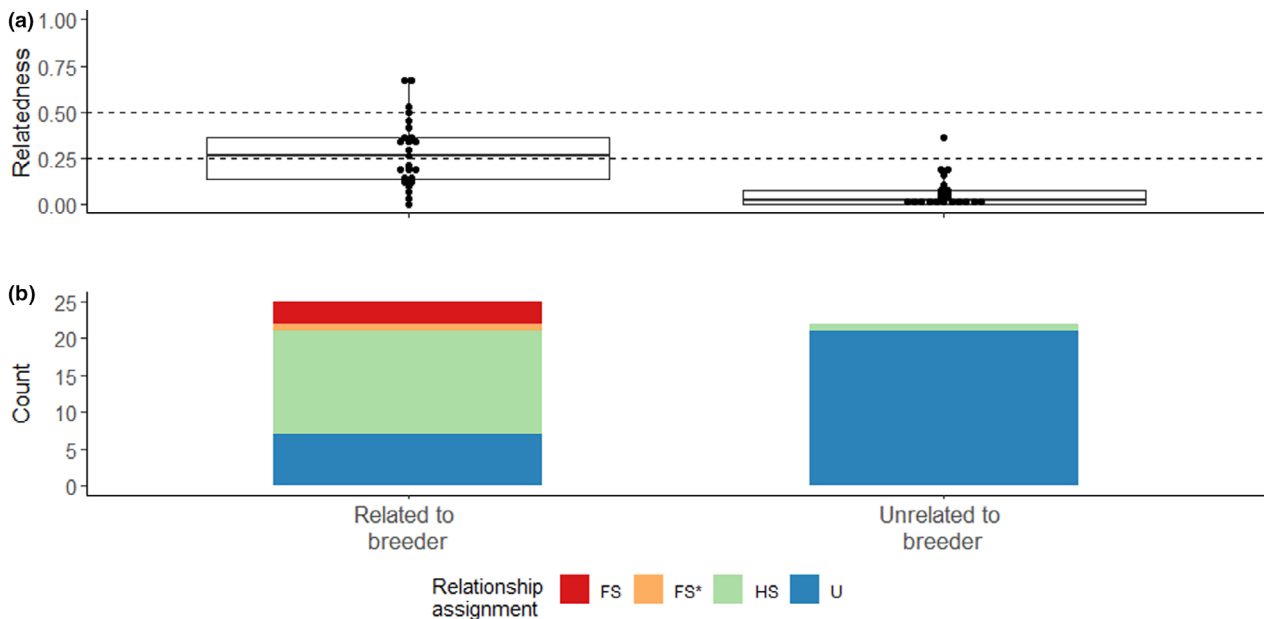


Figure 2. (a) Average relatedness (mean ± se) of helpers to the assisted nestlings according to whether they were genetically related to at least one breeder or not. Dotted lines indicate the expected relatedness coefficients for first-order ($r = 0.5$) and second-order ($r = 0.25$) relatives. (b) Relationships assigned by ML-Relate between helpers and assisted breeders. FS* indicates a full-sibling assigned by observing a helper that could not be genotyped assisting the breeding pair that raised him the previous season. Other details are as in Figure 1.

study, respectively). However, for this individual, we cannot rule out an extra-pair fertilization, which would make the individual a half sibling (consequently it is differentially marked in Fig. 2b). Helpers were estimated as first-order relatives (i.e. *c.* 0.5 coefficient) and full siblings to the nestlings at two nests, and as second-order relatives (i.e. *c.* 0.2 coefficient and half sibling assignment) at another seven nests (Fig. 2), including cases where extra-pair nestlings have been raised. In the nests where we observed extra-pair nestlings, helpers were related only to nestlings sired by the social male. At the remaining eight nests, helpers were unrelated to the raised brood. In one nest with unrelated helpers, one individual was equally assigned as a half sibling and unrelated by ML-Relate. We assumed it was unrelated to the breeding pair and attributed the ambiguous assignment to the unusually large amount of missing data (almost 50% of loci) for this individual. The average estimated level of relatedness was second-order when helpers were genetically related to at least one breeder, whereas the average relatedness corresponded to unrelated when they were non-kin (Fig. 2).

Diverse mating strategies in Baywings

We determined patterns of relatedness and kinship between social parents and nestlings for 31 broods (22 with three or four nestlings and nine with one or two nestlings, Table S1). We included broods with at least three successfully genotyped individuals (between breeders and nestlings). Twenty of those nests (67%) reflected genetic monogamy as all nestlings were sired by the breeding male. Eight nests (22%) had at least one nestling (nine nestlings summing up) that was not sired by the social male and three nests (10%) showed intraspecific brood parasitism (three other nestlings), of which one also had a case of quasi-parasitism. Finally, we found a single case of incest where the breeding female paired with her son, and it was classified as genetic monogamy. This mother–son pair was observed throughout the nesting cycle and confirmed through banding data (the son was banded as nestling at the female's nest; Ursino 2016).

Restricting the analysis to broods of three or four nestlings ($n = 22$), we found that 17 broods (77%) contained full siblings sired by the social parents (genetic monogamy), four nests (18%, five nestlings) had at least one nestling assigned as half

sibling and genetically related to the breeding female but not the male (extra-pair paternity), and one brood (5%) had a nestling that was unrelated to both its nest mates and the social parents, consistent with a case of intraspecific brood parasitism.

Fine-scale genetic structure among Baywings

We detected statistically significant spatial autocorrelation and fine-scale genetic structure in our study population. The global analysis including all individuals showed a significant correlogram ($\omega = 64.2$, $P = 0.01$) with a weak yet detectable pattern up to 300 m ($r = 0.021$, $P = 0.033$, Fig. 3a), where the first distance class presented a higher genetic association than expected by chance ($r = 0.065$, $P = 0.001$, Fig. 3b). A similar pattern was observed among the sampled males, which showed a significant correlogram ($\omega = 80.4$, $P = 0.001$) with a detectable pattern up to 200 m ($r = 0.047$, $P = 0.03$, Fig. 3c), and were significantly more related to neighbours within 100 m ($r = 0.096$, $P = 0.001$, Fig. 3d). The pattern in females was not statistically significant according to our conservative α level of 0.01 ($\omega = 40.4$, $P = 0.02$) and no significant correlation between genetic and geographical distance was found at any point (Fig. 3e,f). Despite the observed differences between male and female spatial data, the overall heterogeneity test did not show significant differences in fine-scale genetic structure between the sexes ($\omega = 42.7$, $P = 0.06$).

DISCUSSION

The genomic analyses presented in this study provide new insights into the cooperative system of the Greyish Baywing. Consistently with earlier work (Fraga 1991, Ursino *et al.* 2017), we found that helping in Baywings is strongly male-biased, a pattern that has also been reported for many other cooperatively breeding birds (Dickinson *et al.* 1996, Russell & Hatchwell 2001). In addition, the analysis presented here showed that two-thirds of the male helpers were genetically related to the individuals they assisted, either as previous offspring of the breeding pair, i.e. mostly first-order relatives (possibly sons or brothers), and less frequently second-order relatives (e.g. half-brothers), of the breeding male. By using ddRAD-seq data and increasing our

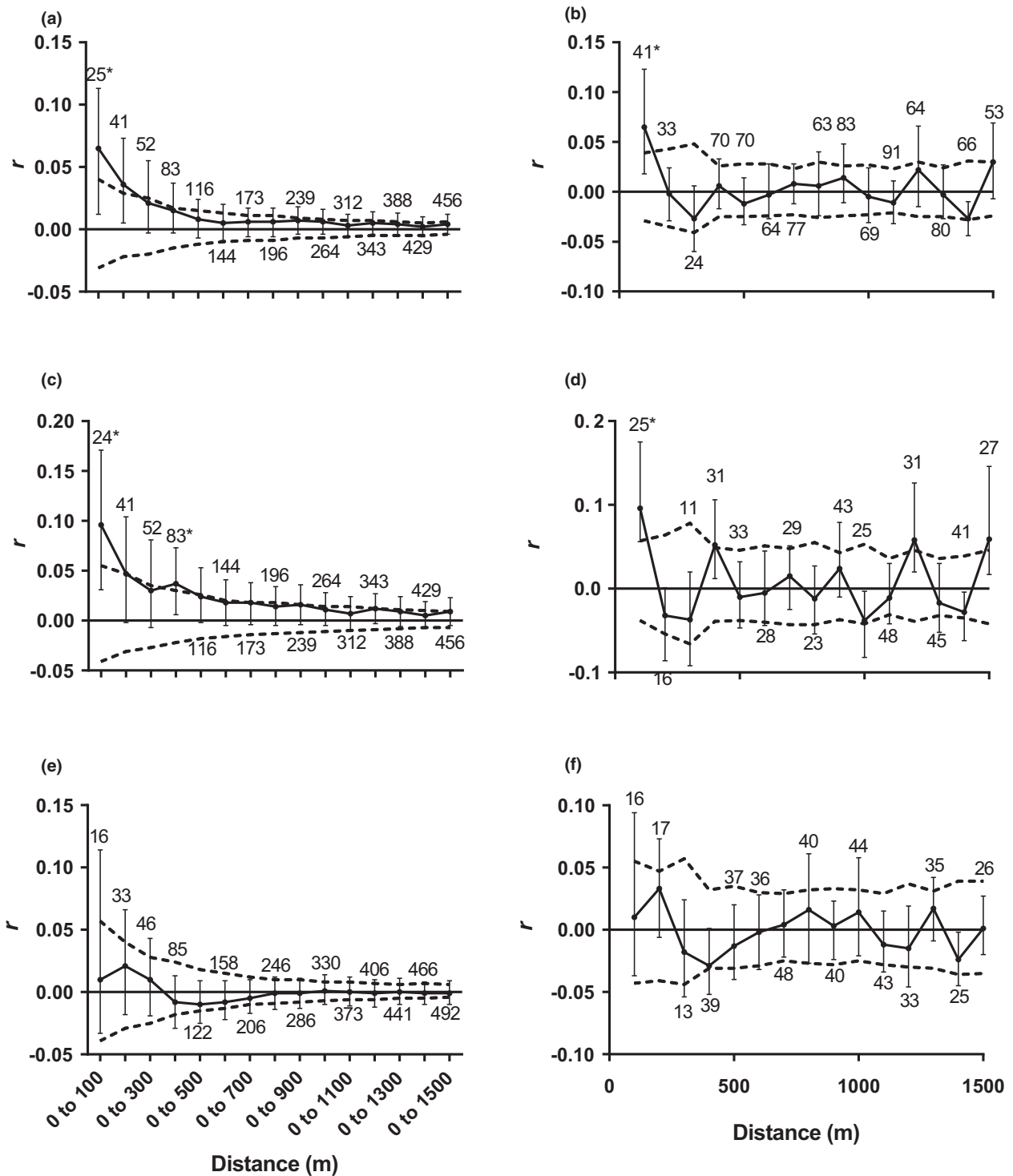


Figure 3. Spatial autocorrelation analysis for adult Baywings excluding helpers. The curves reflect genetic similarity as a function of increasing distance classes (a, c, e) and evenly spaced distance classes (b, d, f) for both sexes (a, b), males (c, d) and females (e, f). The dashed lines indicate 95% confidence intervals under the null hypothesis of no spatial genetic structure in the population, whiskers show the 95% confidence intervals for correlation estimates obtained by bootstrap resampling and asterisks indicate correlations with $P < 0.01$. The numbers above the curve represent the number of pairwise comparisons.

sampling efforts, we were able to advance our understanding of the cooperative breeding system of the Greyish Baywing compared with what was known from previous work (Ursino *et al.* 2017). The relatedness patterns observed in our study could arise through natal philopatry of males that assist their parents and may remain as helpers after the eventual replacement of the breeding female (Dickinson *et al.* 1996, Hatchwell & Komdeur 2000, Double & Cockburn 2003, Ridley 2016). Alternatively, it could be the result of post-dispersal recruitment of male helpers closely related to the dominant male (Russell & Hatchwell 2001, Baglione *et al.* 2003). In either case, the resulting social group composition is compatible with the hypothesized scenario of kin selection influencing cooperative breeding. However, the prevalences of extra-pair nestlings and unrelated helpers suggest that non-reproductive direct benefits may also be at play (Dickinson & Akre 1998). More detailed life history and pedigree information is needed to know the origin of unrelated helpers precisely, but based on our knowledge we propose that they are either non-dispersing extra-pair nestlings (as seen in the Superb-fairy Wren *Malurus cyaneus*, Dunn *et al.* 1995) or adult floaters queuing for breeding turnover (as in the Pied Kingfisher *Ceryle rudis rudis*, Reyer 1986). It might also be that juvenile Baywings occasionally disperse into unrelated social groups that later tolerate them as subordinate adults (as suggested in the Stripe-backed Wren *Campylorhynchus nuchalis*, Piper *et al.* 1995), although empirical support for this last possibility is still lacking.

Kinship patterns between adults and nestlings further indicate that helpers do not gain parentage within their social groups, supporting the occurrence of high reproductive skew, as found in many cooperatively breeding birds with kin-directed helping (Dickinson & Akre 1998, Covas *et al.* 2006, Nelson-Flower *et al.* 2011, Riehl 2017). Reproductive skew towards breeders was strong even in social groups with non-related helpers. In agreement with previous estimates (Ursino *et al.* 2017), we found frequent extra-pair paternity in this study (c. 30% of the nests had extra-pair young). The *related* package estimations were generally consistent with the ML-Relate kinship assignments, although we had low power to discriminate among some parent-offspring and full-sibling comparisons (see Supplementary material, Fig. S2), probably as a result of allelic dropout

(Flanagan & Jones 2019). Despite this lack of fine resolution, we can be certain that helpers did not sire any assisted nestlings. Genotyped nestlings were successfully assigned as offspring of the breeding pair in cases of genetic monogamy in all but one nest, and in this remaining nest, full-sibling assignments were close to half-sibling relationships (log likelihood < 1). Only one of the helpers in our sample sired an extra-pair nestling. This exceptional male helper was assigned as the genetic father (parent-offspring assignment by ML-Relate) of an extra-pair nestling of another breeding group (where it did not help). The nest where this nestling was born started 2 months earlier than the nest where the helper assisted, and they were about 700 m apart. These results suggest that direct genetic benefits do not play a role in promoting helping behaviour in our study population. A similar pattern was observed in the Western Bluebirds *Sialia mexicana*, which show high reproductive skew and moderate to high levels of extra-pair paternity (Dickinson & Akre 1998, Dickinson *et al.* 2016). Under this scenario, kin-directed helping can still be favoured if helpers increase the chances of engaging in extra-pair copulations for genetically related male breeders, for example, through load-lightening (Dickinson *et al.* 2016). However, it is not possible to rule out that helpers were gaining non-genetic benefits, such as benefits derived from philopatry or group-living (Koenig *et al.* 1992, Sorato *et al.* 2015, Shen *et al.* 2017, Kingma 2018, Nelson-Flower *et al.* 2018, Guindre-Parker & Rubenstein 2020). Over the course of the present study we were able to record a few transitions from helping to breeding status, involving four males that were banded as nestlings, helped in their first year and were later re-sighted as breeders. It might be possible that assisting others allows helpers to gain experience for their own future reproduction (Skutch 1961, Komdeur 1996, Hatchwell *et al.* 1999, Komdeur *et al.* 2016) or improve their chances of reaching breeding status (Woolfenden & Fitzpatrick 1984, Piper & Slater 1993, Ribeiro *et al.* 2012). Testing these alternatives is not straightforward, but long-term studies that quantify the survival and reproductive success of helpers while controlling for group size and territory quality would be useful to assess whether helping *per se* results in direct fitness benefits (Dickinson & Hatchwell 2004, Cockburn *et al.* 2016).

The existence of non-reproductive direct benefits of helping could explain the prevalence of

unrelated male and female helpers at Baywing nests (Ursino *et al.* 2017, this study). Alternatively, helping by unrelated individuals could be a cost to be paid for being tolerated in a social group ('Payment of Rent' hypothesis; Gaston 1978, Mulder & Langmore 1993, Bergmüller *et al.* 2005). However, we have not found any evidence from field observations that Baywing helpers suffer punishment or harassment from the dominant pair, regardless of their level of contribution (Rojas Ripari, pers. observ.). The underlying idea is that to stay and help may be the 'best of a bad job' if dispersal and independent breeding are difficult to achieve. As Baywings are cavity nesters, the lack of secondary cavities may lead to shortages in breeding sites, a scenario that has been related to cooperative breeding in species like the Seychelles Warbler *Acrocephalus sechellensis* (Komdeur *et al.* 1995). In comparison to the Seychelles Warbler, our study population is not isolated or spatially restricted, and the lack of nest availability could be compensated for with further dispersion. The study of cavity availability and its effect on cooperative breeding is part of ongoing research; we do not yet have the data to analyse, but hope to address this question in the future. Finally, it is also possible that helping by unrelated males was a side-effect of extra-pair paternity, provided that social groups in Baywings form mainly through delayed offspring dispersal. Following this idea, it could be that at least some unrelated helpers were social (but not genetic) offspring of the breeding male that assisted within their natal group. Increasing our knowledge about the routes to group formation and the fitness consequences of helping would improve our understanding of the non-kin associations in Baywings and, more generally, the stability of cooperation in species with frequent extra-pair matings (Dickinson *et al.* 2016).

One particularly intriguing aspect of the social organization of Baywings is the role of female helpers. In some other cooperative systems, with subordinate females, these can share reproduction through quasi-parasitism or joint nesting (Richardson *et al.* 2002, Riehl 2017, Kaiser *et al.* 2019). This is unlikely to be the case in Baywings because female helpers did not produce any offspring within their social groups (Ursino *et al.* 2017, this study). A possible explanation for the albeit uncommon presence of unrelated female subordinates at Baywing nests is that helping may be a route to inherit a dominant position for females that are queuing for

breeding vacancies (Nelson-Flower *et al.* 2018). Nonetheless, this idea awaits further research.

Male-biased helping could cause sexual differences in dispersal patterns that may result in fine-scale genetic structure. While testing for a spatial association with genetic similarity, we found that male Baywings were more related to their neighbours than would be expected by chance. The genetic structure shown by males was weak and significant only in the shortest distance classes (< 100 m), consistent with dispersal occurring over short distances. The lack of structure over a larger spatial scale suggests annual movements of adult Baywings, as shown in the Ground Tit *Pseudopodoces humilis* where differences in spatial auto-correlations between sexes are present only at the shortest distances, equivalent to the minimum distance among neighbouring breeding pairs (Wang & Lu 2014). Like in Baywings, the authors proposed that the nomadic Ground Tits kept their family ties across seasons despite the persistent turnover in their territories. These results are consistent with field observations showing that adults in our study population were not sedentary between seasons (Fraga 1991). Banded individuals were rarely re-sighted at the same breeding territories over subsequent years, suggesting that territorial behaviour could be rather flexible in Baywings, though keeping family ties. More work is needed to better determine if Baywings show kin associations at the population level. This is important to determine if cooperation among close relatives arises passively as a result of kin structure (Ekman & Griesser 2016) or if potential helpers actively choose to assist close kin (Hatchwell 2016).

CONCLUSIONS

Our genomic data indicate that helping behaviour in Baywings is male-biased and frequently directed to close kin, although some helpers are not related to the groups in which they provide help. In addition, the study supports a strong reproductive skew towards the dominant pair, suggesting that helpers do not accrue direct genetic benefits. Altogether, these results provide novel evidence that is generally consistent with kin-selected cooperation in this Neotropical cooperative breeder. However, as helping by unrelated male and female subordinates was not uncommon, helping behaviour could also be driven by non-genetic benefits such as increased access to resources or breeding opportunities.

Patterns of within-group relatedness and field observations were consistent with male natal philopatry, but our results do not show strong evidence of fine-scale genetic structure in our study population. Future studies that further examine the routes to group formation and the fitness pay-offs of helping behaviour will shed further light on the complex cooperative system of Baywings.

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AUTHOR CONTRIBUTIONS

Juan Manuel Rojas Ripari: Data curation (lead); formal analysis (lead); funding acquisition (supporting); project administration (equal); writing – original draft (lead); writing – review and editing (equal). **Leonardo Campagna:** Methodology (lead); software (lead); supervision (equal); writing – original draft (equal); writing – review and editing (lead). **Bettina Mahler:** Methodology (equal); resources (lead); validation (equal); visualization (equal); writing – review and editing (equal). **Irby Lovette:** Funding acquisition (lead); resources (lead); writing – review and editing (equal). **Juan Carlos Reboreda:** Conceptualization (supporting); project administration (supporting); resources (equal); supervision (supporting); validation (lead); writing – review and editing (equal). **María Cecilia De Mársico:** Conceptualization (lead); funding acquisition (lead); project administration (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal).

CONFLICT OF INTEREST

The authors have no conflicts of interest.

ETHICAL NOTE

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. As the experimental protocols involving the handling of birds were of minimal impact, the University of Buenos Aires committee for animal care and use did not intervene. Manipulations of Baywing nests were performed under permit issued by the local authority (OPDS; 303/16).

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Data Availability Statement

Raw data are available from the NCBI Short Read Archive: <www.ncbi.nlm.nih.gov/bioproject/PRJNA844224>. The subsequent SNP matrix is available in the Supplementary Online data as a text file ([SOM_Appendix1_Genotype-data.txt](#)). See the Supplementary Online Documents for additional data files (Appendixes S1–S5, Figures S1, S2 and Table S1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Genotype_data.txt contains all the analysed samples with the corresponding SNP matrix in related format adapted from genepop.

Appendix S2. Sample_data.xls contains a short description of the breeding status of each sampled individual through the present study and the geographical coordinates (UTM format) in which it was captured and/or banded.

Appendix S3. Relatedness_Helper_Breeder.xlsx contains the pairwise relatedness between (sampled) helpers and the assisted breeding individuals.

Appendix S4. Relatedness_Helper_Nestling.xls contains the pairwise relatedness between the (sampled) helpers and assisted nestlings.

Appendix S5. Relatedness_within_group.xlsx contains the pairwise relatedness between (sampled) breeders and their nestlings.

Figure S1. Example of an agarose gel showing the PCR amplification of the CDH gene.

Figure S2. (a) Boxplots of relatedness values for simulated pairs of known relatedness (FS = full sibling, HS = half sibling, PO = parent-offspring, U = unrelated), and (b) density plot of relatedness values, obtained using the allele frequencies of adult Baywings from the SNP marker panel.

Table S1. Summary data for Baywing individuals genotyped during the study.