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1 BIOLOGICAL SCIENCES

2 **Cost, risk and avoidance of inbreeding in a cooperatively breeding bird**

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13 **Abstract**

14 Inbreeding is often avoided in natural populations by passive processes such as sex-biased
15 dispersal. But, in many social animals, opposite-sexed adult relatives are spatially clustered,
16 generating a risk of incest and hence selection for active inbreeding avoidance. Here we show that
17 in long-tailed tits (*Aegithalos caudatus*), a cooperative breeder that risks inbreeding by living
18 alongside opposite-sex relatives, inbreeding carries fitness costs and is avoided by active kin
19 discrimination during mate choice. First, we identified a positive association between
20 heterozygosity and fitness, indicating that inbreeding is costly. We then compared relatedness
21 within breeding pairs to that expected under multiple mate choice models, finding that pair
22 relatedness is consistent with avoidance of first-order kin as partners. Finally, we show that the
23 similarity of vocal cues offers a plausible mechanism for discrimination against first-order kin
24 during mate choice. Long-tailed tits are known to discriminate between the calls of close kin and
25 non-kin, and they favor first-order kin in cooperative contexts, so we conclude that long-tailed tits
26 use the same kin discrimination rule to avoid inbreeding as they do to direct help towards kin.

27 **Keywords:** Inbreeding, kin discrimination, cooperative breeder, mate choice.

28 **Significance statement**

29 Inbreeding reduces fitness leading to selection for incest avoidance in many organisms. Passive
30 processes, such as sex-biased dispersal, may reduce inbreeding risk, but when dispersal is limited,
31 inbreeding may still be minimized by animals actively recognizing and discriminating kin from
32 non-kin when choosing mates. We investigated inbreeding costs, risk and avoidance in a
33 cooperative bird species in which opposite-sex adults disperse locally to breed and frequently
34 associate. We identified a reduction in fitness in inbred individuals, and show that despite a

35 substantial inbreeding risk, breeders alleviate this by discriminating against close kin as partners.
36 We show that the increased vocal similarity among relatives offers a probable recognition
37 mechanism for this observed level of kin discrimination during mate choice.

38 **Introduction**

39 Inbreeding is generally maladaptive because it increases homozygosity and hence the unmasking
40 of deleterious recessive alleles, which, when expressed, result in a reduction in fitness among
41 inbred individuals termed inbreeding depression^{1,2}. Inbreeding may be tolerated^{3,4}, however, if
42 avoidance is costly, or if the costs of inbreeding are outweighed by the inclusive fitness benefits
43 accrued from breeding with or interacting socially with relatives^{5,6}. Thus, the selection pressures
44 on alternative inbreeding strategies depend on the fitness consequences of inbreeding, typically
45 inferred by the strength of inbreeding depression, and the costs of inbreeding avoidance.
46 Inbreeding depression is often difficult to quantify in natural populations⁷, but it has been shown
47 to select for various avoidance mechanisms^{8,9,10}. Passive processes that disrupt opposite-sex kin
48 associations, such as sex-biased dispersal, are widespread^{11,12}, but when dispersal is constrained¹³
49 or when there is countervailing selection for kin association¹⁴, individuals may frequently
50 encounter kin as potential mates. This is the case in most cooperative breeders, where delayed
51 natal dispersal creates structured populations within which opposite-sex kin associate beyond
52 reproductive maturity¹⁵. In such situations, inbreeding may be minimized by extra-group
53 matings¹⁶⁻²⁰ or by abstention from breeding²¹⁻²³. The latter often results in strong reproductive
54 skew, with reproduction monopolized by a minority of dominant individuals within groups, aided
55 by subordinate helpers²⁴⁻²⁶.

56 Most cooperative species live in discrete groups that occupy exclusive territories, but in some
57 others, helping (providing care to others' offspring) follows local natal dispersal that results in
58 continued association among relatives across extended social networks known as 'kin
59 neighbourhoods'²⁷. Kin neighbourhoods are characterized by a diffuse kin structure where mean
60 relatedness among socially interacting individuals is low. This degree of social organisation also
61 exists in colonial breeders, such as sociable weavers (*Philetairus socius*), in which males and
62 females may recruit as breeders within their natal colony²⁸. Such social structures select for strong
63 kin discrimination in helping behaviour because of the risk of directing care towards non-kin²⁹,
64 and if adult associations include opposite-sex relatives, then strong inbreeding depression would
65 also be expected to select for a mechanism for active incest avoidance.

66 However, the extent to which variation in relatedness across social systems influences inbreeding
67 risk and the strength of kin discrimination exercised during mate choice remain relatively
68 understudied. Fitness costs of inbreeding³⁰ or of being inbred have been identified in several
69 cooperative breeders^{19,20,31}, and active incest avoidance has been demonstrated in western
70 bluebirds (*Sialia mexicana*)³² and inferred in red-winged fairy-wrens (*Malurus elegans*)³³ and
71 grey-crowned babbblers (*Pomatostomus temporalis*)³⁴. But, the discrimination rules used to avoid
72 inbreeding and the recognition mechanisms that effectively minimize its costs have not been
73 determined.

74 Here, we present a comprehensive study of inbreeding depression, inbreeding risk and inbreeding
75 avoidance in long-tailed tits (*Aegithalos caudatus*). Long-tailed tits breed in kin neighbourhoods
76 and exhibit redirected helping, whereby failed breeders acquire indirect fitness by helping to
77 provision non-descendant kin³⁵. Although dispersal is female-biased, natal dispersal distances of
78 both sexes are short³⁶, creating fine-scale genetic structure within breeding populations³⁷. This kin-

79 structure facilitates kin-selected helping, but also results in both kin and non-kin being available
80 as partners when monogamous pairs form each spring³⁸. Using a long-term genetic and life-history
81 dataset³⁹, we assess the evidence for inbreeding depression and a risk of incest, and test putative
82 rules for inbreeding avoidance to determine the likely kin recognition mechanism^{40,41}.

83 **Results**

84 Reduced heterozygosity in inbred individuals is a major source of inbreeding depression, and
85 associations between heterozygosity at microsatellite markers and variation in fitness are widely
86 used as an indirect measure of inbreeding depression when pedigree-derived inbreeding
87 coefficients are unreliable⁴². We tested for an association between standardized heterozygosity at
88 17 microsatellite markers (H) and fitness using four fitness-associated life-history traits: whether
89 an individual recruited to the breeding population; the proportion of eggs that hatched in a female's
90 first clutch; the probability that a breeder produced recruits; and the direct fitness of breeders that
91 produced recruits. Here, direct fitness is a measure of individuals' lifetime reproductive success
92 that corrects for the contribution of helpers (see Methods). This is important because the presence
93 of helpers has a very substantial effect on fledgling recruitment³⁵, and this social effect must be
94 removed to reveal the fitness that most closely reflects an individual's intrinsic 'quality'.
95 Heterozygosity was positively associated with the hatching success of females' clutches (Fig. 1b)
96 and the direct fitness of breeders that produced recruits (Fig. 1d), but there was no association
97 between H and an individual's probability of recruitment (Fig. 1a), nor on the probability that a
98 breeder produced recruits (Fig. 1c). In our analyses, both hatching success and direct fitness are
99 adult traits, and this reduction in fitness of inbred adults indicates that inbreeding has long-term,
100 negative fitness consequences.

101 Long-tailed tits exhibit a significantly enhanced level of relatedness between adult males and
102 females within 600m³⁷, a range within which pairing typically occurs (Fig. 2). However, based on
103 the pedigree, only one out of 609 pairs (0.2%) were first-order relatives, and a further two pairings
104 (0.3%) were between second-order kin (Table S1). Genetic relatedness estimates (r_{QG})⁴³ revealed
105 a similar frequency of close inbreeding (2/609, 0.3%), but substantially more cases of moderate
106 inbreeding (94/609, 15.4%; Table S2). These results suggest active avoidance of close kin when
107 pairing, rather than retrospective extra-pair mating to avoid inbreeding with a related partner.
108 Indeed, the relatively low levels of promiscuity in long-tailed tits^{44,45}, make extra-pair mating an
109 unlikely mechanism of inbreeding avoidance. Instead, we examined whether inbreeding was
110 actively avoided when choosing a social mate.

111 The relatedness of observed pairs was compared with that expected under a series of mate choice
112 models that assumed all first-year, widowed or divorced opposite-sex breeders present in the same
113 year were available as potential partners, within ranges of 300m, 600m, and further 300m
114 increments up to 2100m. Mean r_{QG} to a chosen partner was significantly lower than that expected
115 for females selecting partners at random from within 300m (generalized linear mixed-effects
116 model (GLMM), $n = 2420$, $t = 7.23$, $P < 0.001$), 600m (GLMM, $n = 2433$, $t = 3.93$, $P < 0.001$),
117 900m (GLMM, $n = 2433$, $t = 3.03$, $P < 0.01$), but not 1200m ($n = 2433$, $t = 1.9$, $P = 0.06$; Fig. 3a).
118 Mean r_{QG} to a chosen partner was lower than predicted for males selecting mates from within
119 300m ($n = 2416$, $t = 7.84$, $P < 0.001$), 600m ($n = 2432$, $t = 5.14$, $P < 0.001$), 900m ($n = 2432$, $t =$
120 3.79 , $P < 0.001$) and 1200m ($n = 2432$, $t = 2.54$, $P = 0.01$; Fig. 3b). These results demonstrate
121 strong discrimination against kin as partners within the range that mates are normally chosen,
122 suggesting that inbreeding depression may be sufficiently strong to cause selection for inbreeding
123 avoidance.

124 To identify a plausible discrimination rule for incest avoidance, we compared observed and
125 expected pair r_{QG} assuming either avoidance of first-order kin ($r_{QG} \geq 0.375$), or avoidance of first-
126 and second-order kin ($r_{QG} \geq 0.125$), by removal of these kin from the pool of potential partners at
127 pairing ranges within 1200m. When first-order kin were removed, observed and expected pair r_{QG}
128 did not differ significantly if females selected mates within 300m (GLMM; $n = 2420$, $t = 0.36$, P
129 $= 0.72$), 600m ($n = 2433$, $t = -1.32$, $P = 0.18$), 900m ($n = 2433$, $t = -1.15$, $P = 0.25$) and 1200m
130 ($n = 2433$, $t = -1.46$, $P = 0.14$; Fig. 3a). The same was true for males when they were assumed to
131 select mates from within these ranges (GLMM; 300m: $n = 2416$, $t = 1.47$, $P = 0.14$; 600m: $n =$
132 2432 , $t = -0.05$, $P = 0.96$; 900m: $n = 2432$, $t = -0.29$, $P = 0.77$; 1200m: $n = 2432$, $t = -0.84$, $P =$
133 0.39 ; Fig. 3b). In contrast, when both first- and second-order kin were removed, observed pair r_{QG}
134 was higher than expected at all ranges for both females (GLMM; 300m: $n = 2420$, $t = -9.9$, $P <$
135 0.001 ; 600m: $n = 2433$, $t = -11.46$, $P < 0.001$; 900m: $n = 2433$, $t = -11.52$, $P < 0.001$ and 1200m:
136 $n = 2433$, $t = -11.8$, $P < 0.001$; Fig. 3a) and males (GLMM; 300m: $n = 2416$, $t = -9.16$, $P < 0.001$;
137 600m: $n = 2432$, $t = -11.04$, $P < 0.001$; 900m: $n = 2432$, $t = -11.19$, $P < 0.001$ and 1200m: $n =$
138 2432 , $t = 11.54$, $P < 0.001$; Fig. 3b). Thus, the observed relatedness of breeding pairs closely
139 matches the pattern expected by avoidance of first-order kin as mates. This degree of
140 discrimination can effectively reduce inbreeding because first-order relatives are the category of
141 kin most likely to be encountered nearby in long-tailed tit populations³⁶, although the substantially
142 lower risk of pairing with second-order and more distant kin remains.

143 Long-tailed tits can discriminate kin from non-kin using learned vocal cues⁴¹, a mechanism that is
144 consistent with helpers preferentially aiding close kin^{37,40}. We investigated whether the same
145 mechanism may enable inbreeding avoidance. Our analyses focused on the churr call, a short-
146 range contact call that is highly repeatable within individuals through time⁴⁶. The similarity of the

147 churr calls of opposite-sex breeders varied with relatedness: first-order kin ($n = 20$ dyads) had
148 more similar calls than second-order (GLMM; $n = 249$ dyads, $t = -3.02$, $P = 0.002$) or non-kin
149 (GLMM; $n = 1078$ dyads, $t = -3.62$, $P < 0.001$). Crucially, the calls of males and females within
150 breeding pairs were significantly less similar than those of opposite-sex first-order kin within
151 pairing range (Fig. 4). In contrast, there was no significant difference in vocal similarity between
152 observed pairs and second-order kin or non-kin (Fig. 4). These results suggest that vocal similarity
153 provides a plausible mechanism for avoidance of first-order kin as partners, although we cannot
154 exclude the possibility that other phenotypic cues are also involved.

155 **Discussion**

156 We have shown that inbreeding carries long-term fitness costs in long-tailed tits, but detected no
157 short-term cost on recruitment. Inbreeding depression may be masked in the short-term because
158 external factors such as nest predation have large impacts on offspring fitness in early life.
159 Alternatively, inbreeding depression may affect embryo development or chick survival during the
160 first few days after hatching⁴⁷. We genotyped chicks at 11 days old, so inbred individuals would
161 be a missing fraction in our data if inbreeding depression occurs prior to this age. Furthermore, the
162 probability of both individual recruitment and recruit production are likely to be largely governed
163 by stochastic events, such as predation, whereas hatching success and direct fitness may have a
164 stronger genetic component. The presence of helpers may also mitigate some of the fitness
165 consequences of inbreeding depression. Maternal care buffers inbreeding depression in the burying
166 beetle (*Nicrophorus vespilloides*)⁴⁸, and in long-tailed tits the probability that an individual recruits
167 and its own production of recruits are both correlated with helper number⁴⁹. Investigation into the
168 heritability of life-history traits such as hatching success would further elucidate the mechanism
169 by which inbreeding reduces fitness.

170 Long-tailed tits actively avoid close inbreeding, despite the substantial risk of incest, by avoidance
171 of first-order kin as mates. By contrast, the observed frequency of pairings between second-order
172 kin was relatively high (15.4% of pairs) when using genetic relatedness estimates, although not
173 when using the pedigree (0.3% of pairs). The kin structure of long-tailed tit populations means that
174 after excluding first-order kin, the proportion of birds (of either sex) that are second-order kin
175 within 600m is 14.7% using genetic relatedness estimates and 2.7% of birds using pedigrees³⁷.
176 Thus, our observed frequencies of second-order kin pairings are close to what would be expected
177 from random pairing among birds that are not first-order kin, further supporting our proposed rule
178 for kin discrimination during mate choice (Fig. 3). Together, the significant inbreeding depression
179 and pattern of inbreeding avoidance observed support the hypothesis that there is selection for
180 inbreeding avoidance.

181 These findings are consistent with previous studies demonstrating recognition of first-order kin in
182 a cooperative context^{37,40}. They are also consistent with the idea that kin recognition in long-tailed
183 tits requires a period of association during development, when vocalisations are learned⁴¹. It is
184 very likely that first-order kin (siblings, parents and offspring) associate during rearing, whereas
185 second-order kin are likely to be reared apart. Consequently, vocalisations are more similar among
186 first-order relatives than among second-order or non-kin⁵⁰. There are two instances in which this
187 is not the case: extra-pair paternity and when pair-bonds last more than one year so that full siblings
188 are produced in different nests. However, long-tailed tits are not very promiscuous⁴⁴, and their low
189 mate fidelity across seasons³⁸, high annual mortality and low chance of successful reproduction⁵¹
190 mean that the probability of either instance is low. Avoidance of first-order, but not second-order
191 kin as mates, therefore supports familiarity as the mechanism of kin recognition. However, because

192 long-tailed tits do not live in stable kin groups throughout their life, recognition of familiar
193 individuals still relies on phenotypic rather than spatial cues.

194 Our results suggest that a single kin discrimination rule may explain inbreeding avoidance and kin
195 preference in helping in long-tailed tits, with observational evidence showing that vocal cues offer
196 a plausible mechanism for kin recognition. However, there is an intriguing contrast between the
197 observations that while distant and non-kin are frequently helped³⁵, close inbreeding is extremely
198 rare. A single recognition mechanism can produce variable outcomes depending on the position of
199 the acceptance threshold, which may shift according to the relative fitness costs and benefits
200 associated with acceptance and rejection errors^{52,53}. These in turn will be determined by the
201 probability of encountering a relative and the fitness consequences of the associated behaviour.
202 Assuming that there is some overlap in the similarity of cues produced by close kin and by distant
203 or non-kin⁵⁰ (Fig. 4), an acceptance threshold that includes most close kin, but also some distant
204 or non-kin would explain the observed pattern of helping³⁵. The same recognition threshold could
205 also operate during mate choice but with the reverse effect that almost all close kin, and
206 presumably some distant or non-kin, are rejected as partners, resulting in the infrequent close
207 inbreeding that we observed. A recognition threshold that is generous in the context of helping and
208 stringent in the context of mate choice makes intuitive sense in long-tailed tits. Redirected helping
209 by failed breeders is likely to incur little cost but potentially substantial benefit when kin-
210 directed³⁵. In contrast, inbreeding depression (Fig. 1) suggests selection for strict avoidance of
211 close kin as partners. Therefore, we conclude that a single kin discrimination mechanism has
212 evolved to serve two functions, driving kin association in one context and kin avoidance in the
213 other.

214 **Materials and Methods**

215 **Study population:** A population of 17-72 (mean *c.*50) pairs of long-tailed tits was studied during
216 the breeding season (February-June) between 1994 and 2017 in the Rivelin Valley, Sheffield, UK
217 (53°38'N 1°56'W). The site is approximately 2.5km² and comprises predominantly deciduous
218 woodland and scrub. The population is open: approximately 40% of breeders hatched in the study
219 site and are referred to as native, while the remaining immigrant adults are assumed to have
220 dispersed into the study site during their first year, based on the observation that individuals have
221 high site fidelity following their first breeding year⁴⁹. Each year, almost all individuals (> 95%)
222 were marked with a British Trust for Ornithology (BTO) ring and a unique combination of two
223 color rings. Native birds were ringed as 11-day old nestlings and immigrant adults were captured
224 in mist nests under BTO license before or during their first breeding season. When ringed, a sample
225 of 5-30µl of blood was taken by brachial venipuncture under Home Office license. All breeding
226 attempts were closely monitored and GPS coordinates were taken for each nest (*n* = 1461); a
227 Universal Transverse Mercator coordinate system (UTM) coordinate system was used to describe
228 geographic distance between nests. An ethical review of licensed procedures was undertaken by
229 the University of Sheffield's ASPA Ethical Review Process (Project Applications and
230 Amendments Sub-Committee).

231 **Social pedigree:** We used the social pedigree to predict the correlation between heterozygosity
232 and individual inbreeding coefficients, and to identify matings among known kin in our population.
233 The pedigree was created using 23 years of field observations (1994-2017, *n* = 3068 birds). For
234 further details on pedigree construction, see³⁷. To calculate social relatedness (*r*) among dyads, an
235 additive relationship matrix was generated from the pedigree in R (version 3.5.0, 2018) using the
236 *nadiv* package⁵⁴. Six breeding birds in our study population (0.2%) were from cross-fostered
237 broods in 1996-1998, but given that birds raised together treat each other as kin⁴¹, we included

238 them in the social pedigree. For the same reason, while there is a low rate of extra-pair paternity
239 (11% chicks in 30% nests) in long-tailed tits⁴⁴, it has not been corrected for in the social pedigree.

240 **Inbreeding coefficients:** Inbreeding coefficients were calculated from the social pedigree. It was
241 possible to infer reliable f values from the pedigree for 129 birds (native individuals with all
242 grandparents known). f values from an additional nine birds that were offspring of presumed
243 immigrant siblings, based on genetic sibship reconstruction, were also included. As more distant
244 shared ancestors than grandparents, if known, would cause individual inbreeding coefficients to
245 increase, f values are likely to be under-estimated based on incomplete pedigree information.

246 **Molecular genetics:** Individuals were genotyped at 17 microsatellite loci⁵⁵. Population allele
247 frequencies were generated in CERVUS (version 3.0.7, 2007). All available genotypes were used
248 (1994-2017, $n = 3304$ birds) to maximize accuracy and ensure non-zero estimates for all alleles.
249 The genetic relatedness of dyads, r_{QG} , is a genetic estimate of the coefficient of relatedness based
250 on genetic markers was estimated using coefficient of relatedness estimates (43), calculated in
251 SPAGeDi (version 1.1.5, 2002). This estimate is reliable when tested against our social pedigree⁵⁶.

252 **Inbreeding:** Inbreeding cases were identified using the social pedigree and genetic relatedness
253 estimates. Genetic (r_{QG}) and social (r) relatedness of all breeding pairs from 1994-2016 in which
254 both adults were ringed and genotyped was calculated. Measurements were taken from distinct
255 pairs. Occasionally, long-tailed tits swap partners within a breeding season, in which case, the first
256 pairing of that year was used. Individuals often breed in multiple years, either with the same partner
257 or a new partner. The dataset used in this study contained 609 pairs made up of 445 females and
258 412 males in 1994-2016. Pairs were considered closely or moderately inbred if they comprised
259 known first-order ($r = 0.5$) or second-order ($r = 0.25$) kin, respectively. As incomplete social

260 pedigrees may underestimate incest rates in open populations, inbreeding was also quantified using
261 genetic relatedness estimates (r_{QG}). The r_{QG} estimate of known first-order kin ($r = 0.5$) was 0.468
262 ± 0.136 (mean \pm SD, $n = 500$ dyads). For known second-order kin ($r = 0.25$), r_{QG} was $0.241 \pm$
263 0.179 (mean \pm SD, $n = 338$ dyads). The r_{QG} estimate of all other dyads of known parentage ($r <$
264 0.25) was 0.004 ± 0.133 (mean \pm SD, $n = 25638$ dyads). The distribution of r_{QG} estimates among
265 known first-order, second-order and non-kin are shown in Fig. S1. Based on these distributions, a
266 lower r_{QG} threshold of 0.375 was set to approximate first-order kin (mean $r_{QG} \pm$ SD = $0.502 \pm$
267 0.094 , $n = 1148$) and 0.125 to approximate second-order kin (mean $r_{QG} \pm$ SD = 0.197 ± 0.059 , n
268 = 9926). The mean r_{QG} of observed pairs was 0.002 ± 0.123 (mean \pm SD, $n = 609$).

269 **Mate choice models:** For each focal breeder, their r_{QG} to their chosen partner was compared with
270 their mean r_{QG} to all potential partners, each breeding year (1994-2016), under the pairing
271 constraints of a series of mate choice models assuming all first-year, widowed or divorced
272 opposite-sex breeders present in the same year were available as potential partners, within
273 concentric ranges of radius 300m, 600m, 900m, and further 300m increments up to 2100m.

274 **Heterozygosity-fitness correlations:** Pedigree-derived inbreeding coefficients can be estimated
275 only when parentage can be traced back at least two generations, but both sets of grandparents
276 were known for only 5.3% of native birds ($n = 138$). Therefore, standardized multi-locus
277 heterozygosity (H) was estimated for all genotypes (1994-2016, $n = 3182$). Heterozygosity is
278 standardized by dividing the proportion of typed loci for which an individual was heterozygous by
279 the mean heterozygosity of those loci at which the individual was typed⁵⁷. Heterozygosity-fitness
280 correlations can only be regarded as providing evidence for inbreeding depression if
281 heterozygosity is a predictor of individual inbreeding coefficients. We used the analytical
282 derivations outlined in³⁹ to predict the correlation between heterozygosity and f in our population

283 as $r(H, f) = -0.43$ ($n = 138$, mean $f = 0.03$, variance in $f = 0.004$, number of loci = 17, mean
284 heterozygosity of loci = 0.759). This value is relatively large compared to other studies predicting
285 the relationship between inbreeding coefficient and heterozygosity, including populations where
286 inbreeding depression has been demonstrated. For example, the correlation coefficient $r(H, f)$ in
287 red deer (*Cervus elaphus*)⁵⁸ and song sparrows (*Melospiza melodia*)⁵⁹ are -0.25 and -0.22,
288 respectively³⁹. Thus, genetic diversity at marker loci reflects genetic diversity throughout the
289 genome, including at unknown loci that affect trait variation; i.e. marker and fitness loci are in
290 identity disequilibrium⁴². This validates the use of heterozygosity as a proxy for inbreeding
291 coefficient in our study. Measurements were taken from distinct samples.

292 **Direct fitness:** Direct fitness was calculated as lifetime reproductive success quantified in terms
293 of genetic offspring equivalents and corrected for extra-pair paternity and the offspring gained by
294 having helpers. The fraction of recruits in a brood that was attributable to helpers was estimated
295 using a mixed effects model of the effect of helper number on recruitment⁴⁹. This fraction was
296 subtracted from the total number of recruits produced over an individual's lifetime. The remaining
297 fraction was halved to reflect the relatedness between a single parent and its offspring. The
298 assumption that parents and their offspring have a relatedness coefficient of 0.5 does not account
299 for higher relatedness of inbred offspring to their parents⁶⁰. However, the almost complete absence
300 of close inbreeding and the low incidence of inbreeding among more distant relatives indicate that
301 errors in our estimation of direct fitness introduced by this simplifying assumption will be small.

302 **Acoustic recordings:** A short-distance contact call, the churr, was recorded from adults using a
303 Sennheiser ME67/K6 shotgun microphone fitted with a Rycote windjammer. Recordings were
304 made onto a Roland R-05 version 1.03 WAV/MP3 recorder with a 6GB SanDisk memory card,
305 set to a sample rate of 48kHz with WAV-16bit accuracy. The microphone input level was set to

306 60db with a low-cut frequency of 400Hz. All recordings were made between 06:00 and 18:00 BST.
307 Birds were recorded at a distance of approximately 3-15m, to minimize sound degradation and
308 reverberation. Birds were recorded at the nest and identified by their unique color ring
309 combinations. If more than one bird was present, vocalizations were assigned to individuals by
310 observing movements of the bill and throat feathers. At the start of each recording, date, time, nest
311 number and recording number were dictated into the microphone. When caller ID could be
312 identified with certainty, this was dictated into the microphone after each call. In total, 213
313 recordings were made in 2015-2017, containing 1116 churr calls from 98 birds (mean \pm SD =
314 11.39 ± 10.24 per bird; range 1 – 42).

315 **Acoustic analysis:** The sampling frequency was converted to 22.05 KHz and recordings were
316 visualized spectrographically to assess call quality, with a frequency resolution of 188Hz and a
317 time resolution of 2.7ms in Avisoft SAS-Lab Pro version 4.52 (Avisoft Bioacoustics). Recordings
318 with extreme background noise were excluded. All useable calls were isolated, stored and
319 measured in Luscinia (version 2.16.10.29.01, <https://rflachlan.github.io/Luscinia/>). Vocal
320 similarity was assessed by dynamic time-warping analysis (DTW) implemented in Luscinia. DTW
321 analysis generates a score representing the amount of warping required to match one signal to
322 another. The acoustic features used in the DTW analysis were weighted as: time = 1, fundamental
323 frequency = 2, change in fundamental frequency = 2, compression factor = 0.1, minimum element
324 length = 10, time SD weighting = 1, ArcTan transform weight for frequency slope = 0.02,
325 maximum warp = 100%. These settings generated a DTW algorithm that correctly matched
326 visually similar vocalizations, assessed using a dendrogram and multidimensional scaling plot.
327 The low compression factor optimizes the capture of acoustic complexity. This increased
328 weighting of frequency parameters to time is also in line with previous studies suggesting that

329 frequency parameters show greater individuality than temporal parameters and are particularly
330 important for kin recognition in this species⁴⁶.

331 **Call similarity and pairing:** Among the breeding pairs for which we had recordings of both
332 breeders ($n = 51$), there were no cases of pairing among known first-order or second-order kin,
333 based on the social pedigree. Based on genetic relatedness estimates, there were no cases of pairing
334 among first-order kin ($r_{QG} \geq 0.375$) and 13 (25.5%) cases of pairing among second-order kin (r_{QG}
335 ≥ 0.125). Dyadic vocal similarity (DTW score) was compared among: breeding pairs; potential
336 pairs of first-order kin ($r_{QG} \geq 0.375$); potential pairs of second-order kin ($0.375 > r_{QG} \geq 0.125$) and
337 potential pairs of non-kin ($r_{QG} < 0.125$) within 1350m, the range within which 95% pairs are
338 formed. Genetic estimates of pedigree relationships were used for consistency with our analysis of
339 putative discrimination rules. Potential pairings were dyads of opposite-sex first-year, widowed or
340 divorced breeders present in the breeding population in the same year. The distance between adults
341 was based on the location of an individual's first breeding attempt in a given year.

342 **Statistical analysis:** All statistical analyses were carried out in R (version 3.5.0, 2018).
343 Associations between heterozygosity and fitness traits (HFCs) were tested using generalized linear
344 mixed-effects models in the lme4 package. Recruitment was modelled as a binary response
345 variable with a binomial error distribution and logit link. The fixed effects were: H, sex, to control
346 for male-biased philopatry, fledge date (days since March 1st), because offspring fledging earlier
347 in the year have a greater probability of recruitment⁶¹, and number of helpers at natal nest, as helper
348 number has been shown to increase recruitment probability⁵¹. Hatching success was modelled as
349 a proportional response variable with a binomial error distribution and logit link. The fixed effects
350 were: H, lay date and female mass as a nestling. The probability of producing recruits was
351 modelled as a binary response variable with a binomial error distribution and logit link. The fixed

352 effects were: H and fledgling sex ratio (proportion of male fledglings produced, to control for male
353 philopatry). Direct fitness was modelled as a continuous response variable with a Gamma error
354 distribution and inverse link, with H, sex and fledgling sex ratio fitted as fixed effects. In all HFC
355 models, genetic brood was fitted as a random effect to avoid pseudoreplication of H estimates and
356 control for seasonal differences. In hatching success models, breeding year was also fitted as a
357 random effect.

358 Analyses of the mating options available to males and females were conducted in separate mate
359 choice models. As the same allele frequencies are used to calculate r_{QG} across years, the r_{QG} of
360 unique dyads across years is consistent. However, due to demographic factors such as divorce,
361 migration, birth, death, and dispersal, the mean r_{QG} of focal breeders to their potential partners
362 under each mate choice model will vary across years. To quantify inbreeding avoidance and
363 identify a putative decision rule with regard to kinship, we fitted linear mixed-effects models with
364 restricted maximum likelihood. For focal males and females, we compared r_{QG} to chosen partner
365 with (i) mean r_{QG} to potential partners under random mate choice (with respect to kinship), (ii)
366 mean r_{QG} to potential partners after the removal of close kin, and (iii) mean r_{QG} to potential partners
367 after the removal of close and distant kin, within pairing ranges of 300m, 600m, 900m and 1200m.
368 When pairs persisted across years, the first year a pair was observed was used in the analysis. Year
369 nested within focal bird ID was fitted as a random effect, to generate comparisons within
370 individuals in a given year.

371 Churr call dissimilarity was compared among four groups of individuals (breeding pairs, potential
372 breeding pairs of first-order kin, potential breeding pairs of second-order kin and potential
373 breeding pairs of non-kin) using generalized linear mixed-effects models. DTW score was
374 modelled as a continuous response variable with a Gamma distribution and inverse link function.

375 The fixed effect was group, with male ID and female ID both fitted as random effects. The
376 relationship between churr call dissimilarity and kinship was tested using a separate model that
377 included all genotyped breeders, irrespective of pairing status. . DTW score was modelled as a
378 continuous response variable with a Gamma distribution and inverse link function. The fixed effect
379 was kinship, with male ID and female ID both fitted as random effects.

380 **Data availability:** Source datasets and code for this manuscript have been deposited in the Dryad
381 Digital Repository, <https://doi.org/10.5061/dryad.k6djh9w49>.

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391 **Author contributions**

392 BJH conceived and managed the long-tailed tit study and supervised the project with EJHR. AEL,
393 BJH, SPS and MS designed the study and collected data. AEL performed all analyses and wrote
394 the manuscript. JS supported analysis of inbreeding costs. RFL supported bioacoustic analysis.

395 JPG calculated direct fitness. All authors contributed to revisions. The authors declare no
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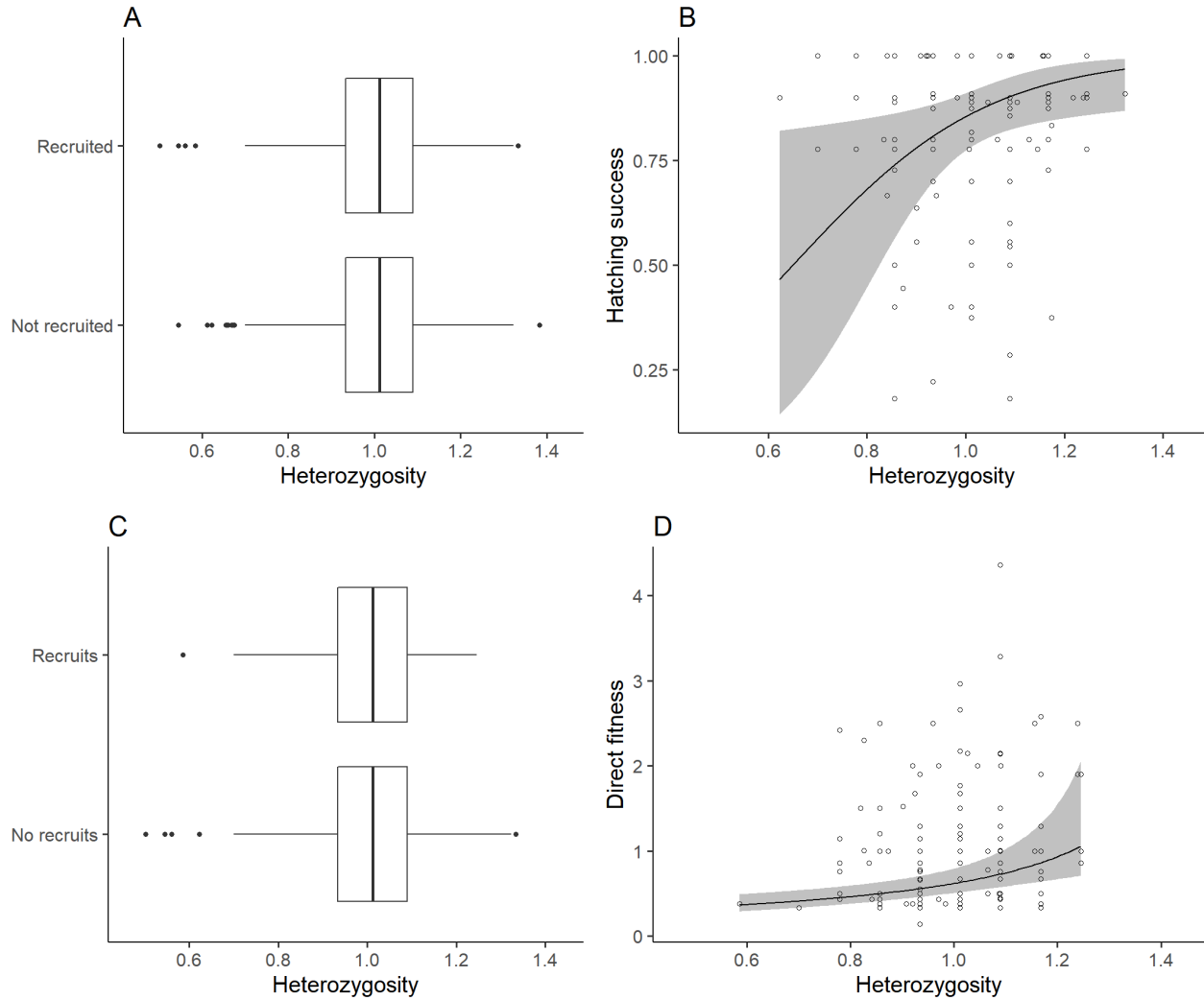
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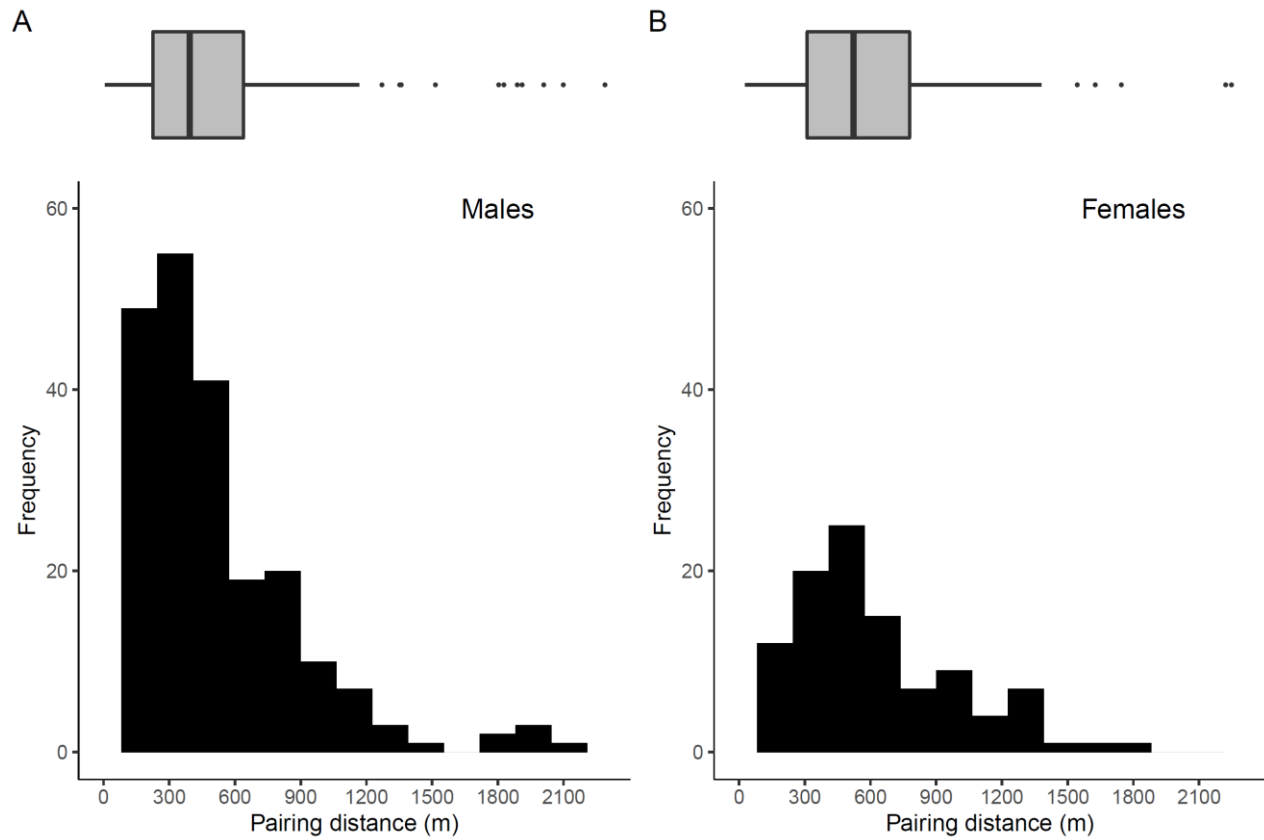
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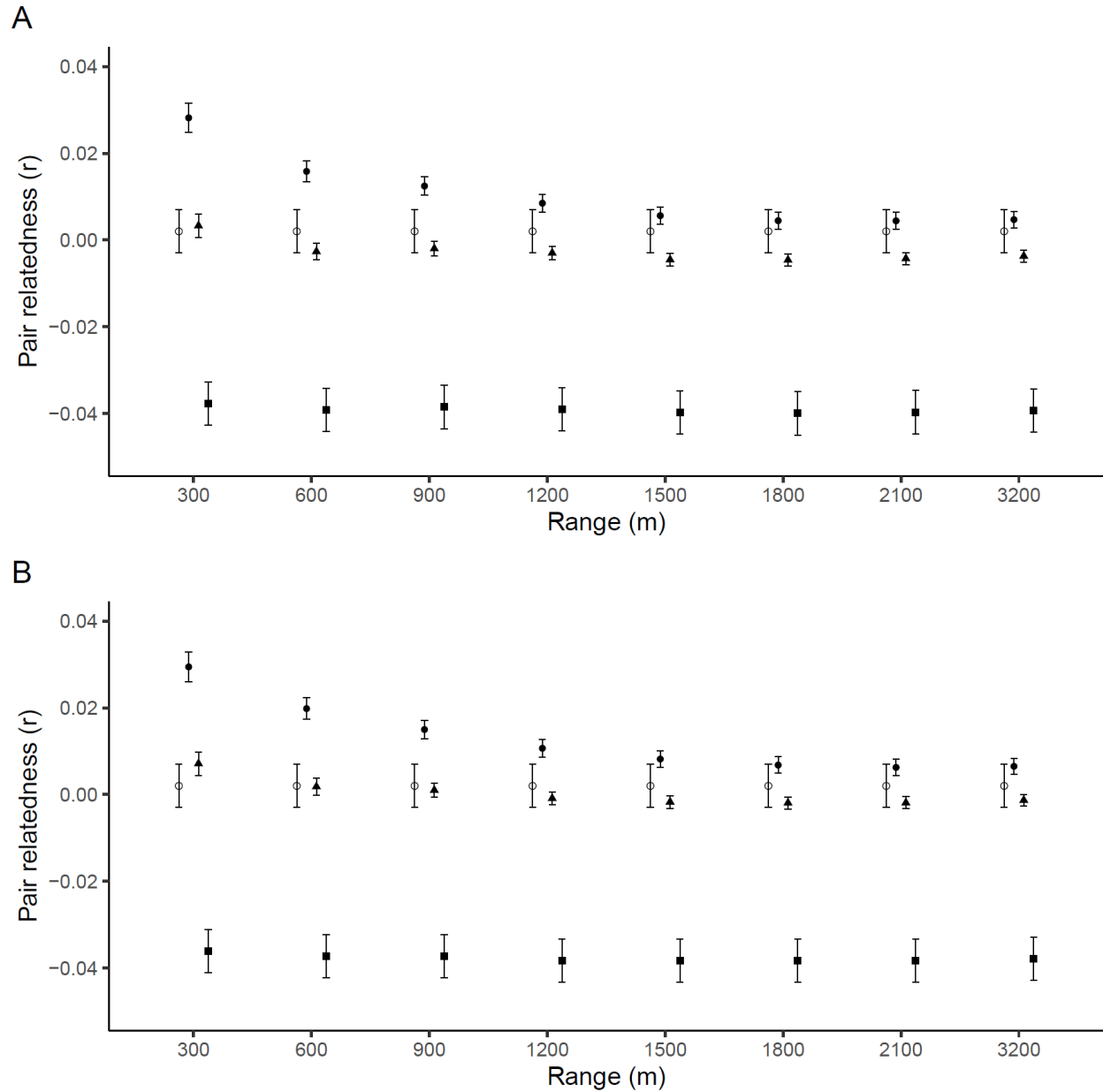
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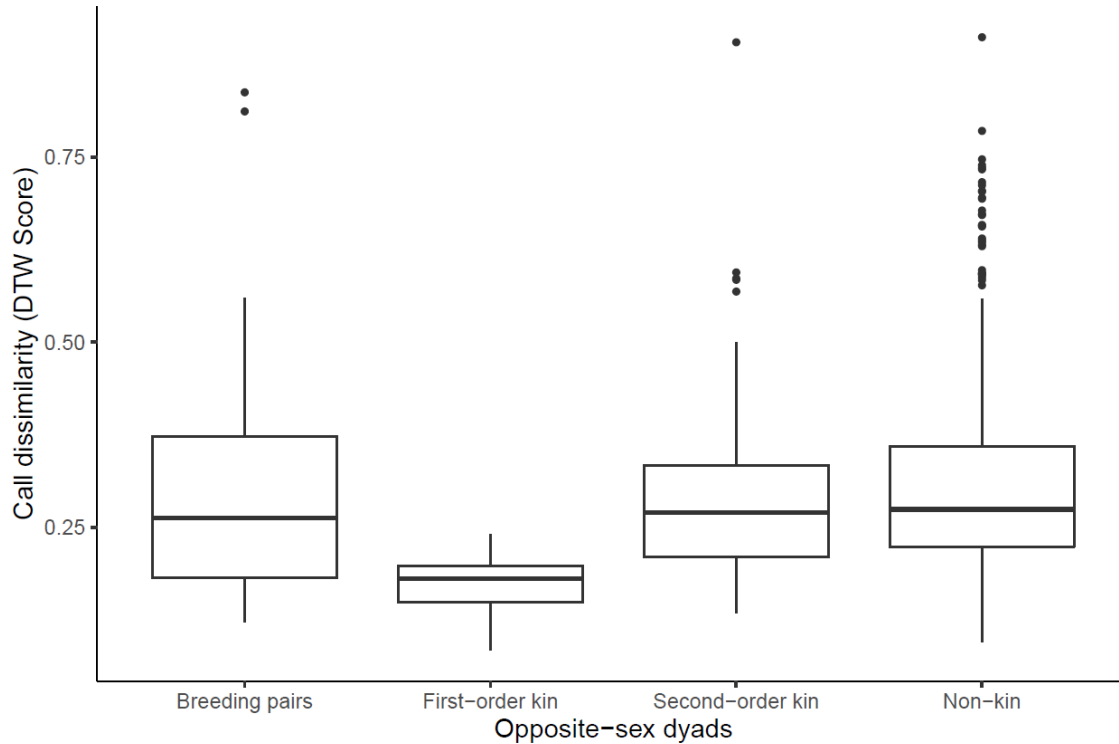
544 **Fig. 1.** The relationship between heterozygosity at microsatellite loci (H) and fitness components.
 545 (A) probability of recruitment was not associated with H (GLMM, $N = 1924$, $z = 0.40$, $P = 0.69$);
 546 (B) females' hatching success was positively associated with H (GLMM, $N = 142$, $z = 2.32$, $P =$
 547 0.02); (c) probability of producing recruits was not associated with H (GLMM, $N = 744$, $z = -1.77$,
 548 $P = 0.07$); (d) the direct fitness of breeders that produced recruits was positively associated with
 549 H (GLMM, $N = 151$, $t = -4.65$, $P < 0.001$). Full model outputs are reported in Tables S3-6. Lines
 550 represent model predictions $\pm 95\%$ CI constructed using fixed effects, boxplots represent median
 551 $\pm 1.5x$ IQR.



552 **Fig. 2.** Frequency distribution and median (+IQR) pairing ranges of (A) male (median = 393m, N
 553 = 230) and (B) female (median = 523m, N = 109) breeders, calculated as the distance between an
 554 individual's natal nest and their first breeding attempt. Boxplots represent median \pm 1.5x IQR.



555 **Fig. 3.** Mean genetic relatedness of breeding pairs formed within increasing ranges (open circles),
 556 and the expected relatedness if (A) females ($N = 445$) or (B) males ($N = 412$) selected mates at
 557 random with respect to kinship (closed circles), avoided kin with $r_{QG} > 0.375$ (closed triangles),
 558 and avoided kin with $r_{QG} > 0.125$ (closed squares). Expected relatedness was the mean relatedness
 559 of focal birds to all opposite-sex available breeders within each range under each mate choice
 560 model. Error bars represent the standard error around the mean.



561 **Fig. 4.** Dissimilarity of churr calls among groups of opposite-sex dyads: breeding pairs ($N = 51$);
 562 first-order kin ($N = 11$); second-order kin ($N = 155$); non-kin ($N = 735$). Dyads that were not
 563 breeding pairs comprised available breeders within pairing range ($\leq 1350\text{m}$, 95% pairs) present in
 564 the same breeding year. Call dissimilarity was measured using dynamic time warping analysis
 565 (DTW). Call dissimilarity within breeding pairs was higher than that within potential pairs of first-
 566 order kin (GLMM; $N = 952$, $t = 2.87$, $P = 0.004$) but not second-order ($t = 0.06$, $P = 0.94$), or non-
 567 kin ($t = -1.63$, $P = 0.10$). Boxplots represent median ± 1.5 IQR.