Use of microsatellite-based paternity assignment to establish where Corn Crake *Crex crex* chicks are at risk from mechanised mowing

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APPENDIX S1

SUPPLEMENTARY METHODS

Study area

The Nene Washes (52.58°N, 0.07°W) is a canalised section of the River Nene in Cambridgeshire, England, UK. It was built as a flood protection structure during the drainage of the marshlands of the Fenland Basin. The 15 km^2 strip of wet grassland, up to about 1 km wide, parallel to the river, is designated as a Site of Special Scientific Interest under the Wildlife and Countryside Act 1981 because of its aggregations of breeding and non-breeding birds and its ditch and grassland flora. It is also a site designated under the Ramsar Convention on Wetlands, a Special Area of Conservation under Article 3 of the European Union's Habitats Directive and a Special Protection Area under Article 4 of the European Union's Birds Directive. Corn Crakes ceased to breed at the site early in the 20th Century. Part of the site is owned and managed as a nature reserve by the RSPB. Since 2004,

the Nene Washes has a re-introduced breeding population of Corn Crakes located in a section of the grassland strip about 7 km long.

Singing male survey and capture methods and interpretation

Adult male Corn Crakes produce a loud rasping 'crek-crek' song almost continuously on most nights when they are not in a short-term (7–10 days) pair bond with a female (Tyler & Green 1996). Survey routes, traversed using a vehicle, were planned to approach within 300 m of all areas of tall grass and herbs potentially suitable for Corn Crakes. Surveys were conducted between 22:00 and 03:00 BST at intervals of 2 – 4 days on nights with suitable weather conditions. Rain and windy conditions (Beaufort Force 5 or more) were avoided. Co-ordinates of the locations of singing males were first identified by triangulating the sound from mapped listening points. Singing places were later approached on foot to within 50-100 m and their locations determined using the mapped locations of features such as ditches, bushes and gateways and a hand-held GPS (Garmin eTrex 10). All of the singing locations used to calculate distances between capture localities of young Corn Crakes and the singing sites of their potential fathers were known to within 20 m.

Night-time records of singing males were assigned to individuals using their locations and whether or not sets of males were recorded singing on the same night. We began the process of assigning records to individuals by identifying as separate individuals males that were singing on the same night, beginning with the night when the maximum number was counted. The presence of individuals additional to this set was identified by capturing them to read or apply BTO rings.

Singing males were captured at night by luring them into nearby mistnets using a broadcast recording of conspecific song. Their capture locations were determined using a hand-held GPS. In the absence of capture evidence to the contrary, we assumed that night-time singing records within 200 m of a capture location were of the ringed individual caught there, but we often made further captures to check this. We attempted to capture all the males detected on night-time surveys and, in doing this, we captured some males more than once, with 14 being captured twice and three on three occasions during the course of the same breeding season. Recaptures usually occurred when a singing male was heard in an area where no male had been caught previously in the season, but capture revealed that it was an individual already captured elsewhere which had moved. A few males evaded repeated attempts at capture throughout the breeding season, but we consider that these were identifiable as unique and separate individuals with reasonable confidence, based upon their evasion behaviour and locations.

We were unable to estimate the number of adult females present at the Nene Washes because there is no method available for surveying them. However, we note that equal numbers of adult males and adult females were captured on July–August drives (Main text: Table 1), so the number of adult females in the population was probably similar to the counts of singing males.

Validation of estimated numbers of singing males

The accuracy of our assessment of the total number of singing males present depends upon whether we correctly identified as separate individuals the males we were unable to catch whilst they were singing. To check this we performed a mark-recapture analysis of data collected by the same methods as those described here, but obtained over a longer period

68 (2004–2018) than is considered in this paper. From all ringing and recapture records of adult
69 males from this period, we identified the Manly-Parr set of observations (Manly & Parr
70 1968), each of which refers to a male-year in which an individual was known to be alive
71 because it had been recorded in a previous year and also in a subsequent year. The Manly-
72 Parr set comprised 16 male-years involving 15 males in 6 focal years (2008, 2009, 2010, 2014,
73 2016 and 2017). The male was captured whilst singing in 15 of the 16 male-years (annual
74 probability of capture = 15/16 = 0.938, binomial confidence limits, 0.698 – 0.998). If our
75 method for assessing the total number of singing males is accurate, we would expect that
76 this annual probability of capture derived from mark-recapture analysis present would be
77 the same as the ratio of the number of individuals captured to the total estimated present.
78 For the six Manly-Parr focal years, the mean of the ratio of the number of males captured to
79 the total estimated was 0.824 (95% confidence limits, 0.651 – 0.997). This analysis indicates
80 that the ratio of minimum number of individuals known present from captures to our
81 estimates of the number of singing males present was similar to, and not significantly
82 different from, the expectation based upon the mark-recapture estimate of the annual
83 probability of capture. In our study period, all five of the adult males captured during drives
84 in July–August, after the end of the singing season, had already been captured earlier in the
85 same year as singing males (Main text Table 1). Combining both of these lines of evidence,
86 we are confident that we captured and sampled a high proportion of the potential fathers of
87 the chicks we sampled.
88
89 **Drive catching and sampling of adults, chicks and juveniles**
90 Corn Crake adults, chicks and juveniles were captured by driving them into funnel traps,
91 similar to Ottenby traps (Bub 1991), made from flexible plastic netting (Cintoflex M, Tenax

92 UK Ltd, Wrexham, UK). For each drive, an approximately rectangular area of 1.2–4.7 ha of
93 tall grass and herbage was enclosed by a combination of fences of plastic netting and
94 existing barriers, such as water-filled ditches. Corn Crakes within it were driven into a line
95 of traps set approximately equally spaced at one end of the drive area and linked by drift
96 fences. Further details of the method are given by Green (2010).

97 A slow (<200 m/h) drive was made by a team of people towards the trap-line from
98 the opposite end of the drive area, using tractor noise generated by MP3 players and
99 disturbance of the ground vegetation by dragging a 2.5 cm diameter polypropylene rope
100 over it. In one instance, when downy chicks estimated to be seven days old were seen and
101 heard calling during a drive, the number in the brood was estimated by eye and only one
102 was captured by hand, to reduce disturbance. Traps were checked periodically and the
103 captured birds were placed in cloth bags. The assumed location before disturbance of the
104 young chick captured by hand was the actual capture location because chicks as young as
105 this move slowly in response to disturbance (Tyler *et al.* 1998) and produce loud calls when
106 separated from their mother (Green *et al.* 1997b). In all other cases, the brood location before
107 disturbance occurred was taken to be the centre of the drive area. Although the true
108 locations of broods before the disturbance caused by the drive would probably have been
109 more uniformly distributed within the drive area than this, we assumed that the centre of
110 the drive area was a reasonable approximation of the mean of undisturbed positions when
111 calculating the distance of chick locations to the singing place of their father. However, to
112 assess the sensitivity of our conclusions about the chick-father distances to failure of this
113 assumption, we also measured the shortest and longest distances between any part of the
114 drive area in which a chick was captured and the father's singing place. Further details of
115 the catching method are given by Green (2010). The age of captured young was estimated

from measurements, using established methods described below. Buccal swab samples were collected. Chicks and juveniles were released in the drive area close to the trap in which they were caught. Where probable mothers were caught with young, they were released together.

Determining the age of chicks

Captured chicks and juveniles were distinguished from adults following Salzer & Schäffer (1997). All birds, except the chick of seven days old, were marked with uniquely numbered BTO metal rings. Body weight, maximum chord wing length and the length of the waxy sheath on the growing 7th primary (numbering descendantly from proximal to distal) were measured Green & Tyler (2005). Young of the year, with no waxy sheath on the 7th primary, were classed as fully-grown juveniles. Other young were classed as unfledged chicks. The age of chicks weighing less than 109 g was estimated from the body weight and that of heavier chicks from the ratio of the length of the waxy sheath to the maximum chord wing length, by the method of Green & Tyler (2005).

The hatching date of a group of chicks of similar age (< 3 days different), identified as siblings from the microsatellite results, was estimated by subtracting the mean age of the brood from the capture date. Fully-grown juveniles, not captured previously as chicks, could not be aged using body weight or primary wax, so we assumed that they were 50 days old because primary growth is completed at 45 days old (Green & Tyler 2005) and radio-tagged juveniles have been found to depart from the natal area soon after this (Donaghy *et al.* 2011). The first-egg date of the clutch from which a brood was derived was taken to be 26 days before the hatching date, assuming eight days as the laying period of a typical clutch and 18 days as the incubation period (Green *et al.* 1997b).

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141 **DNA sampling and extraction and parentage assignment**

142 The mouth of each captured bird was swabbed using a sterile cotton swab on a wooden stick
143 (Sterilin F150CA) rotated gently against the buccal epithelium anterior to the base of the
144 tongue 20–30 times. The swab was then replaced in its plastic protective sheath. Within a
145 few hours of sampling, the cotton bud was cut off the stick and stored in a tube containing
146 sufficient 100% ethanol to immerse the bud.

147

148 **DNA preparation and genotyping**

149 Genomic DNA was extracted from the buccal swabs using an ammonium acetate method
150 (Richardson *et al.* 2001). The DNA samples were then genotyped for 15 microsatellite loci
151 (Gautschi *et al.* 2002, Brede *et al.* 2010, Dawson *et al.* 2010) and one sex marker (Dawson *et al.*
152 2015), which were run in three multiplex groups (Table S1). Polymerase chain reactions
153 (PCR) were run in a total volume of 10 μl, which contained: 5 μl multiplex PCR mix (Qiagen
154 Inc., Valencia, USA), 2 μl ddH_2O, 1 μl fluorescently labeled primer-mix and 2 μl extracted
155 DNA. The PCR program (Veriti Thermal Cycler - Applied Biosystems) was: 95°C for 15 min,
156 then 40 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 60 s, followed by a final 60°C for 30
157 min. Fluorescent-labeled PCR products were analyzed on a 3730 DNA Analyzer (Applied
158 Biosystem, California, USA), and allele sizes were scored using GENEMAPPER 4.0 (Applied
159 Biosystems) and a GeneScan 500 ROX size-marker (Applied Biosciences).

160

161 **Parentage assignment**

162 We assigned parentage first using a Bayesian approach, in R 3.2.2 (R Core Team 2017), using
163 the package *MASTERBAYES* 2.52 Hadfield *et al.* 2006) and then in COLONY 2.0.3.3 (Wang

2013). We used 14 microsatellites; we excluded *Crex11* as some samples showed three peaks using GENEMAPPER, and this marker had high null allele frequencies in other Corn Crake populations (Fourcade *et al.* 2016).

We tested for Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD) using GENEPOP 4.2 (Rousset 2008). For the HWE and LD analyses, we removed potential relatives using COANCESTRY 1.0.1.7 (Wang 2011) to choose pairs with zero relatedness based on TrioML. We selected individuals that had the highest number of zero relatedness (≥18) with other individuals in the populations, which resulted in 31 individuals.

Seven loci deviated from HWE: *Crex6*, *Crex8*, *Crex12*, *N3B3*, *Crex2*, *TG02-120*, and *TG04-012*. These deviations may arise from the small dataset (N=31) and the presence of some relatives. We therefore also conducted HWE tests on the same 14 loci from 28 captive bred Corn Crakes, from which birds introduced into the Nene Washes population originated. Four loci deviated from HWE (*Crex6*, *Crex1*, *Crex2* and *TG04-012*) and only two of these were the same as in the Nene Washes population. Furthermore, of 210 HWE tests from 15 Corn Crake populations using the same loci, (except for *N3B3*), 23 deviated from HWE (Fourcade *et al.* 2016), but the loci differed across populations.

After False Discovery Rate (FDR) control (Benjamini & Hachberg 1995), to account for multiple tests, four pairs of loci were in LD: *Crex8* & *TG04*-041, *TG04-041* & *TG12-015*, *Crex9* & *TG04-012* and *TG12-015* & *TG05-030*. We also conducted LD tests on the captive bred birds; after FDR control, two pairs of loci were in LD (*Crex6* & *Crex8*, and *Crex8* & *TG12-015*), but these differed to those in the wild population. No deviations from LD were detected across 15 populations using the same loci, (except for *N3B3*) by a previous study (Fourcade *et al.* 2016), so all 14 loci were retained in our analyses.

Parentage was assigned in *MASTERBAYES* using allele frequencies extracted from all 81 genotyped birds from the Nene Washes population, and a default allelic drop-out and stochastic error rate of 0.005. The number of unsampled mothers and fathers were estimated by *MASTERBAYES* and no restrictions were placed on the number of tolerated mismatches between parents and offspring. Paternity assignments were weighted by the Euclidian distance between the candidate father (N = 43) and offspring (N = 31), and both parents were sampled simultaneously. Maternity assignments were not weighted by distance as only five adult females were genotyped and models containing this parameter did not converge. We ran 130,000 iterations, saving every 100th and discarding the first 30,000, to ensure autocorrelations between successive parameter estimates were <0.1. Metropolis-Hastings acceptance rates were checked to lie between the acceptable range of 0.2 and 0.5 (Hadfield *et al.* 2006).

We then assigned additional sibships for offspring born in 2013 and 2014, using COLONY. We specified the parents already assigned with a probability ≥0.80 from *MASTERBAYES*. We assumed monogamy for males and females, a probability of 0.2 of either the mother of father being in the candidate mother or father pools, and an error rate of 0.01.

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Table S1. Details of the three multiplexes (MP) used to analyse the 15 microsatellite loci and one sexing locus, along with their fluorescent label, primer concentrations and reference sources.

Locus	MP	Fluorescent label	Primer concentration in primermix μM)	Source
Crex6	1	Hex	0.5	Gautschi *et al.* (2002)
Crex9	1	Ned	0.5	Gautschi *et al.* (2002)
Crex7	1	Fam	0.5	Gautschi *et al.* (2002)
TG04-041	1	Hex	0.25	Dawson *et al.* (2010)
Crex8	1	Fam	0.5	Gautschi *et al.* (2002)
TG012-015	1	Hex	7.5	Dawson *et al.* (2010)
Z37B_sex	2	Fam	0.25	Dawson *et al.* (2015)
Crex11	2	Hex	0.5	Gautschi *et al.* (2002)
Crex12	2	Ned	0.5	Gautschi *et al.* (2002)
N3B3	2	Fam	0.5	Brede *et al.* (2010)
TG04-012a	2	Hex	0.5	Dawson *et al.* (2010)
Crex2	3	Hex	0.5	Gautschi *et al.* (2002)
TG04-012	3	Ned	0.5	Dawson *et al.* (2010)
Crex1	3	Fam	1.5	Gautschi *et al.* (2002)
TG05-030	3	Hex	0.5	Dawson *et al.* (2010)
TG02-120	3	Fam	0.5	Dawson *et al.* (2010)

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