Spatiotemporal patterns of egg laying in the common cuckoo

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Understanding egg-laying behaviour of brood parasites in space and time can improve our knowledge of interactions between hosts and parasites. However, no studies have combined information on the laying activity of an obligate brood parasite with detailed information on the distribution of host nests within an area and time period. Here, we used molecular methods and analysis of egg phenotypes to determine maternal identity of common cuckoo, *Cuculus canorus*, eggs and chicks found in the nests of four species of *Acrocephalus* warblers in consecutive years. The median size of a cuckoo female laying area (calculated as a minimum convex polygon) was correlated negatively with the density of host nests and positively with the number of eggs assigned to a particular female. Cuckoo female laying areas overlapped to a large extent and their size and location did not change between years. Cuckoo females preferentially parasitized host nests located close to their previously parasitized nests and were mostly host specific except for two that parasitized two host species. Future studies should focus on sympatric host and parasite communities with variable densities across different brood-parasitic systems to investigate how population density of hosts affects fitness and evolution of brood parasites. For instance, it remains unknown whether female parasites moving to new sites need to meet a threshold density of a potential host. In addition, young females may be more limited in their egg laying, particularly with respect to the activity of other parasites and hosts, than older females.

Key words: Acrocephalus warbler, egg-laying territory, host selection, maternity, minimum convex polygon, spatial analysis

Brood parasitism occurs in birds, fishes, arachnids and insects (Blažek, Polačik, Smith, Honza, Meyer, & Reichard, 2018; Boulton & Polis, 2002; Cervo, Stemmer, Castle, Queller, & Strassmann, 2004; Davies, 2000). It is a reproductive strategy in which the parasites relinquish all parental care to other species, the hosts. In birds, obligate brood parasites lay eggs in multiple nests of one or more host species to maximize their annual reproductive output (Soler, 2017).

The spatiotemporal pattern of egg laying by individual parasitic females is thus a key life history trait that deserves attention (Payne & Sorensen, 2005). In particular, it is essential (1) to identify female laying areas (Langmore, Adcock, & Kilner, 2007; Martínez, Soler, Soler, & Burke, 1998; Strausberger & Ashley, 2003), (2) to explore the consequences of parasitism for individual parasites and their hosts (Koleček et al., 2015; Stokke, Honza, Moksnes, Røskaft, & Rudolfsen, 2002), and (3) to better understand the coevolutionary interactions between the brood parasite and its host (Soler, 2017; Yang et al., 2010).

Radiotracking telemetry and molecular methods have revealed that brood-parasitic females overlap territories rather than defend exclusive areas (Bolopo, Roncalli, Canestrari, & Baglione, 2020; Fleischer, 1985; Martínez et al., 1998; Moskát, Bán, Fülöp, Bereczki, & Hauber, 2019; Nakamura & Miyazawa, 1997; Rühmann, Soler, Pérez-Contreras, & Ibáñez-Álamo, 2019; Ursino, Strong, Reboreda, & Riehl, 2020; but see Langmore et al., 2007). At the same time, both host-specialist and host-generalist individuals exist within various parasite taxa (Alderson, Gibbs, & Sealy, 1999; Ellison, Sealy, & Gibbs, 2006; Nakamura, Miyazawa, & Kashiwagi, 2005; Strausberger & Ashley, 2005; Vogl, Taborsky, Taborsky, Teuschl, & Honza, 2004; Woolfenden et al., 2003) and individual parasitic females may return to the same home range between years (Hahn, Sedgwick, Painter, & Casna, 1999; Hauber, Heath, & Tonra, 2020; Koleček, Procházka, Brlík, & Honza, 2020). In addition, there is evidence for nonrandom selection of host nests for parasitism (Mahler, Confalonieri, Lovette, & Reboreda, 2007), which vary according to the particular host community that is being parasitized (De Mársico, Mahler, Chomnalez, Di Giacomo, & Reboreda, 2010).

The common cuckoo, *Cuculus canorus* (hereafter cuckoo) is an obligate brood parasite of a broad range of host species (Davies 2000), although individual females are specialized in particular host species (Fossøy et al., 2016; Marchetti, Nakamura, & Gibbs, 1998; Stokke et al., 2018; but see Vogl et al., 2004). Cuckoos were previously thought to have distinct breeding

territories (Dröscher, 1988) but recent radiotelemetry work has shown that, similarly to other parasitic species, they maintain overlapping breeding territories of extremely variable size (Moskát, Elek, Bán, Geltsch, & Hauber, 2017; Vogl et al., 2004). A more detailed analysis of the tracking data also revealed that females appear to overlap in the use of host individuals (Nakamura & Miyazawa, 1997; Vogl et al., 2004). However, radiotelemetry can provide only incomplete information on the spatiotemporal distribution of egg laying. Video recordings revealed that cuckoo egg laying takes from a few seconds (Jelínek, Šulc, Štětková, & Honza, 2021; Wang, Zhong, He, Zhang, & Liang, 2020) to several minutes (Moksnes, Røskaft, Hagen, Honza, Mork, & Olsen, 2000) and therefore, the egg-laying process needs continuous tracking which cannot be provided by radiotelemetry. A step forward is to use genetic analysis of parasitic offspring to estimate the spatiotemporal patterns of egg-laying behaviour in individual cuckoo females.

Here we used molecular and egg phenotype analysis to investigate the distribution of egg laying in the cuckoo and its changes throughout two consecutive breeding seasons. We combined information on laying with data on the distribution and timing of host nests within the same area and time period in four regularly parasitized hosts breeding in sympatry in the southeastern part of the Czech Republic (Edvardsen, Moksnes, Røskaft, Øien, & Honza, 2001). Specifically, we localized the parasitized nests belonging to two major hosts, great reed warbler, *Acrocephalus arundinaceus* (hereafter GRW) and Eurasian reed warbler, *A. scirpaceus* (RW) and some nests of sedge warbler, *A. schoenobaenus* (SW) and marsh warbler, *A. palustris* (MW).

In line with current knowledge, we predicted that (1) the high cuckoo density and parasitism rate observed in our study area (Honza, Požgayová, Procházka, & Koleček, 2020) will lead to a high overlap between female laying areas (Vogl et al., 2004). The size of the laying areas should naturally depend on the availability of host nests (Langmore et al., 2007). Therefore, we

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predicted that (2) large laying territories of cuckoo females will occur in areas with a low density of host nests. Subsequently, as cuckoos (Koleček et al., 2020) and their hosts (Koleček et al., 2015) are faithful to their breeding sites, (3) we did not predict any significant change in the position of laying areas between years (Vogl et al., 2004). Furthermore, we predicted that (4) most cuckoo females will parasitize just one host species (Fossøy et al., 2011; Honza et al., 2002). Finally, before laying an egg, cuckoo females spend a considerable amount of time near the host nest chosen for parasitism (Honza et al., 2002). Therefore, we predicted that (5) following a parasitism event, they will be more likely to lay their next egg in a host nest close to the previously parasitized nest provided the neighbouring nest is suitably timed.

<H1>METHODS

<H2>Fieldwork

The study was carried out in the fishpond complex and adjacent wetland habitats near Mutěnice (48°54′N, 17°02′E) and Hodonín (48°51′N, 17°07′E) in the Czech Republic from May to July 2016 and 2017 (Fig. 1). Upon arrival of GRW males, we extensively mapped their territories daily over the entire study site and thus assumed we found annually almost all GRW nests including renesting after previously unsuccessful breeding attempts (see Table 1 for numbers of nests). The proportion of nests found belonging to RW was slightly lower (approximately 80%, exact percentage unknown) and the better concealed nests of SW and MW were found only occasionally (Table 1). However, the abundance of the latter two species and thus also the absolute number of their nests parasitized by cuckoos within the region is relatively low in the long term (see also Edvardsen et al., 2001; Kleven, Moksnes, Røskaft, Rudolfsen, Stokke, & Honza, 2004). We recorded the geographical position of all parasitized nests in every species and all GRW nests using GPS (Garmin Oregon 300 Pro).

Most GRW nests were found during the building stage. The rest of the GRW nests and most nests of the other three host species were found at different stages of breeding. To record the laying date of cuckoo eggs, we checked individual GRW nests almost every day until host clutch completion. Afterwards, we visited the nests less often (typically every 3–4 days) until the end of the breeding attempt (for details see Honza et al., 2020). All nests of the other host species were checked approximately every second day during the laying stage and occasionally during incubation. In 2016, we also continuously filmed the majority of GRW nests at the study site during the egg-laying stage to record all parasitism events (for details see Jelínek et al., 2021).

DNA samples of cuckoo offspring were collected as follows. Since only one cuckoo chick usually survives in the host nest, we removed the newly laid (second and following) cuckoo eggs from multiply parasitized nests. As DNA from the freshly laid eggs could not effectively be sampled, we transferred them to an incubator (HEKA-Kongo; HEKA-Brutgeräte, Rietberg, Germany) and incubated them artificially until hatching (we then placed chicks into host nests for purposes of other studies). When chicks were 10 days old, we took a blood sample (approximately 25 µl) from their ulnar or medial tarsometatarsal vein. For unhatched chicks and chicks that died before the age of 10 days we used tissue samples instead. We also mist-netted 20 adult cuckoo females and took blood samples (approximately 25 µl). All DNA samples were stored in 96% ethanol until later genetic analyses.

Identity of Cuckoo Mothers: Molecular Determination

DNA was extracted from blood or tissue samples using Tissue Genomic DNA mini kit (Geneaid Biotech Ltd, New Taipei, Taiwan). To infer the identity of cuckoo mothers, we used multiple genetic markers: single nucleotide polymorphisms (SNPs) and mitochondrial markers.

First, we genotyped all samples with the ddRAD (double digest restriction-site associated DNA) technique (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) following the protocol of Piálek, Burress, Dragová, Almirón, Casciotta, & Říčan (2019) to acquire the SNP data set and determine individual identity. The sample libraries were sequenced on an Illumina HiSeq4000 system (two lanes, 150 cycles P/E) in the European Molecular Biology Laboratory Genomic Core Facility, Heidelberg, Germany. The RAD-tags were processed in Stacks v2.4 (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Rochette, Rivera-Colón, & Catchen, 2019) and mapped on the cuckoo genome GCA000709325.1 (https://www.ncbi.nlm.nih.gov) with Bowtie2 assembler v2.2.4 (Langmead & Salzberg, 2012). Only loci with 95% or higher presence of individuals were scored. The loci were further filtered in PLINK v1.9 (Purcell et al., 2007) so that only loci in Hardy–Weinberg equilibrium that did not show evidence of linkage disequilibrium and with alleles with a minimum frequency of 0.4 were used (--hwe 0.01 'midp' --indep 100 10 1.2 --maf 0.4) which resulted in a data set with 1620 variants.

Second, for the mitochondrial haplotype analysis, we sequenced a 411-bp portion of the left-hand hypervariable control region (Fossøy et al., 2011; 2012; Gibbs, Sorenson, Marchetti, Brooke, Davies, & Nakamura, 2000). Mitochondrial sequence data were assembled and manually checked in Geneious v10.2.6 (Kearse et al., 2012) and haplotypes were estimated based on a distance matrix with up to 1% tolerance (approximately four mutations) for genotyping errors.

Finally, we determined the identity of cuckoo mothers for individual offspring using the program Colony (Jones & Wang, 2010). Colony enables identification of individual offspring and determines their half- and full-sibling relationships using a full-pedigree likelihood approach and also allows the inclusion of additional information about known relationships among the offspring to increase the probability of correctly assigning sibling relationships (i.e. based on mtDNA in our case).

168 Parentage was estimated based on > 1000 nuclear SNPs supplemented with mitochondrial DNA

169 haplotypes enabling us to exclude highly implausible maternal (or maternal – sibling)

170 relationships in the inferred genealogy. None of the eggs assigned to a cuckoo female were laid

171 on the same or subsequent days, which agrees with cuckoo laying intervals of around 48 h

172 (Nakamura et al., 2005; Seel, 1973; Wyllie, 1975).

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174 <H2>Identity of Cuckoo Mothers: Phenotypic Determination

175 We expanded our data set of all genetically assigned offspring by eggs, whose phenotypes were

176 measured but could not be genetically assigned to cuckoo females because no DNA was

177 sampled due to early host ejection or predation. For this purpose, we developed software

178 evaluating the visual similarities of particular cuckoo eggs. We analysed colour pattern and

179 colour dimension data from calibrated photographs and spectrophotometry data (for details see

180 Šulc et al., 2019). Spectral data of the background colour was obtained using spectrophotometry

181 measurements. Shape data obtained from the photographs included the length, maximum width,

182 volume, ellipse deviation and surface area of the eggs. For pattern data we used custom scripts

183 to calculate pattern energies and skew metrics that gave measures of how patterned the eggs

184 were and the spatial distributions of the patterns. Finally, luminance data were analysed using

185 the photographs, including both the spots and background areas of the eggs. In the first step,

186 we employed principal component analyses (PCA) of all visual metrics (separately for each

187 year) to reduce collinearity and the number of variables (Šulc et al., 2020a.). In total, we used

188 11 egg phenotypic characteristics resulting from PCA (namely two PCA components from

189 spectral data, a measurement of the mean brightness, the position of the UV peak, three PCA

190 components from shape data, three PCA components from pattern data and one PCA

191 component from luminance data; see Šulc et al., 2020a for further details). In the second step,

we trained a random forest model (R package randomForest; Liaw & Wiener, 2002) using a subset of genotyped eggs to label pairs of eggs as 'same' or 'different' (Šulc et al., 2020a). The random forest method is an ensemble learning method where many decision trees are constructed during training that allow assignment to a class (in this case, same or different), and then assignment for each row of data is based on the mode of the classes for individual trees. The training set used 3000 'same' rows, where the two eggs were laid by the same cuckoo female (but were not phenotypically identical to each other) and 3000 'different' rows, where the two eggs were laid by different females. To test our model, we examined each egg in the labelled data set on all eggs sequentially, including itself. We first tested whether the model recognized the identical eggs as being the same. As the model was not trained on these comparisons, this served as a check for the accuracy of the model. We then tested whether each egg was paired only with other eggs from the same female, i.e. whether the model could uniquely identify clusters of eggs that belonged together. The entire process (creating a training set, training the random forest model and testing the model) was repeated 1000 times (Šulc et al., 2020a).

For the eggs without genetic information, we calculated how many times in each of these 1000 runs the target egg was matched with a cluster of eggs laid by the same female. If the percentage was higher than 95%, we considered this egg as a candidate for being from this female. To corroborate this conclusion, we used nonphenotypic criteria: (1) two cuckoo eggs of one female could not be laid on the same or subsequent days and (2) the cuckoo female had to be the same colour morph (rufous or grey; data from video recordings). If we found at least one of the other criteria did not meet the requirements, we did not include this egg in our analyses. None of the criteria were violated and thus we did not exclude any of the final set of 38 phenotypically assigned eggs.

Laying Areas and Timing of Egg Laying

Based on molecular and phenotypic determination and for each year separately, we defined individual laying areas as minimum convex polygons (MCP; R package adehabitatHR; Calenge, 2006), joining the outermost nests parasitized by each cuckoo female and calculating the area.

The laying date of each cuckoo egg was expressed as the most probable date on which the egg was laid. A newly found cuckoo egg might have been laid either on the day it was first recorded in a host nest or on the day before (Gärtner, 1981; Honza et al., 2020; Sealy, 1992). In 48 cases, we knew the exact time of parasitism (mainly from continuous video monitoring and occasional observations in situ). Except for three cases, the nests were parasitized after midday (see also Honza et al., 2020; Moksnes et al., 2000). Therefore, the laying dates of cuckoo eggs found before 1200 hours CEST were estimated as the mean of the day before the date it was first recorded in a host nest and the day of the last check before it was first recorded. Laying dates of the eggs found after 1200 CEST were estimated to be the mean of the date of recording and the day of the previous check.

Data Analysis

All analyses were performed using pooled molecular and phenotypic data. We calculated the spatial overlap of each cuckoo laying area with other laying areas overlapping in time using R packages geosphere, mapview, raster and rgeos (Appelhans, Detsch, Reudenbach, & Woellauer, 2018; Bivand & Rundel, 2017; Hijmans 2017; 2019). We calculated orthodromic distances (package birdring; Korner-Nievergelt & Robinson, 2015) between the centroids (i.e. average positions) of the nests parasitized by cuckoo females between the 2 years (including females with one or two offspring).

We also tested whether the size of a cuckoo laying area reflects the density of host nests during her laying period. We expressed the density as the number of active GRW nests per ha of laying area belonging to cuckoos parasitizing GRWs and related the density to the size of laying area using a Spearman rank correlation coefficient.

To elucidate whether cuckoo females preferentially parasitize nests close to previously parasitized host nests, we first calculated the orthodromic distance between two GRW nests consecutively (i.e. after 2 days, see above) parasitized by one cuckoo female. Using a Wilcoxon paired test, we compared these distances with the median distances to all GRW nests suitable for parasitism at the same time. We considered GRW nests suitable for parasitism within the first 4 days of egg laying since most cuckoo females parasitize within this period as later the probability of early cuckoo hatching decreases in GRW (Geltsch, Bán, Hauber, & Moskát, 2016; Honza et al., 2020). All statistical analyses were performed in R 3.4. (R Core Team, 2018).

Ethical Note

This study was carried out with the permission of the regional nature conservation authorities (permit numbers JMK: 115874/2013 and 38506/2016; MUHOCJ: 41433/2012/OŽP, 34437/2014/OŽP, and 14306/2016/OŽP). The fieldwork adhered to the animal care protocol (experimental project numbers 039/2011 AV ČR and 3030/ENV/17-169/630/17) and to the Czech Law on the Protection of Animals against Mistreatment (licence numbers CZ 01272 and CZ 01284). All work complies with the ASAB/ABS guidelines for the treatment of animals in research.

During mist-netting and blood sampling, adults and nestlings were held for less than 10 min and the amount of blood taken was <1% of body mass. The cuckoo chicks sampled for blood

265 grew normally. No mortality or other adverse effects were observed during capture or blood

266 sampling, and the host nests were not abandoned as a result of territory mapping, mist-netting

267 or egg collection and measurements. All the collected cuckoo eggs were transported in a box

268 lined with cotton wool and kept warm during transport.

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270 **<H1>RESULTS**

271 *<H2>Laying Areas and Timing of Laying*

272 The median number of offspring assigned to one cuckoo female in 1 year was 3 (minimum =

273 1, maximum = 15, $median_{GRW}$ = 3, $median_{RW}$ = 2, N = 27 females in GRW and 31 in RW in

274 2016 and 2017; Fig. 2, Appendix Table A1). The median laying area of a female reached 14.3 ha

275 (minimum = 0.01, maximum = 2 622.2; $median_{2016}$ = 32.3, $median_{2017}$ = 8.2, N = 13 females

276 with a minimum of 3 offspring in 2016 and 19 females in 2017; Table 1, Appendix Table A1).

277 Laying area was strongly positively correlated with the number of offspring per cuckoo female

278 (Spearman rank correlation: r_S = 0.77, P < 0.0001) and laying areas of different females

279 overlapped 0–100% ($median_{2016}$ = 50.8%, $median_{2017}$ = 93.1%; Fig. 1, for individual laying

280 areas see https://isobirdnet.shinyapps.io/Cuckoo_territory/). Moreover, laying area of cuckoos

281 parasitizing GRW nests was strongly negatively correlated with the density of active GRW

282 nests (Spearman rank correlation: r_S = –0.95, P < 0.0001, N = 15 laying areas in both years)

283 and this also held for 10 laying areas located solely on fishponds (i.e. not including surrounding

284 forests and farmland; Spearman rank correlation: r_S = –0.85, P = 0.002).

285 The median distance between the centroids of the laying areas belonging to one female in

286 both study years was 293 m (minimum = 76, maximum = 4 728, N = 11 pairs of laying areas).

287 In addition, laying area did not differ between years (Wilcoxon paired test: V = 40, P = 0.577)

288 and laying date of the first parasitic egg differed by 1–32 days (median = 7.5).

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Cuckoo females laid their eggs between 6 May and 2 July (median = 31 May, N = 233 eggs with known laying date) and the laying date did not differ between eggs laid in GRW and RW host nests (Wilcoxon test: W = 6126, P = 0.436, N = 133 eggs in GRW and 98 in RW nests). The time span between laying the first and last parasitic egg within 1 year varied between 3 and 51 days (median = 25 days, N = 37 females with at least two assigned eggs with known laying date) and was strongly positively correlated with the number of offspring assigned to individual cuckoo females (Spearman rank correlation: r_S = 0.79, P < 0.0001).

Choice of Host Species and Nests

In total, 25 cuckoo females with at least two assigned offspring laid their eggs in the nests of one host species (Table 1, Appendix Table A1). Only two cuckoo females parasitized two host species: one laid one and four eggs in the nests of RW and GRW, respectively and the other laid one and two eggs in the nests of MW and RW, respectively (Appendix Table A1). None of the cuckoo females changed their host species between 2016 and 2017. We found only one case when a female that parasitized GRW in both years also parasitized one RW nest in 2017 (see above).

Cuckoo females preferentially parasitized the nest closest to the nest where they had laid their previous parasitic egg. Namely, in 25 of 55 known cases of two cuckoo eggs subsequently laid into two nests of GRW, the latter was laid closer to the previously parasitized nest than to the other GRW nests suitable for parasitism on the same day. In all but five of the 55 cases, the nests used for parasitism were closer than at least half of the other nests suitable for parasitism on the same day. The median distance between two consecutive parasitic events was 613 m (minimum = 65, maximum = 3657) and was much shorter than the median distance to all GRW

nests suitable for parasitism at the same time (2 532 m, minimum = 65, maximum = 11 395; Wilcoxon paired test: $V = 86$, $P < 0.0001$).

<H1>DISCUSSION

Here we showed that (1) cuckoo laying areas varied considerably in size and overlapped to a large extent. (2) The size of laying areas was negatively related to density of host nests. (3) The size and position of laying areas of females did not vary much between the 2 years. (4) The vast majority of cuckoo females parasitized a single host species and (5) cuckoo females preferentially parasitized nests closest to their previously parasitized nests.

Laying areas found in this study varied greatly in size (median = 14.3 ha, up to 2622.2 ha). Vogl et al. (2004) also reported highly variable sizes of potential laying areas using VHF radiotelemetry at the same site and host species (median = 27.3 ha, up to 179 ha), but their sample was limited ($N = 7$) and radiotelemetry could not reliably detect all cases of parasitism or fully distinguish laying from feeding areas (see also Nakamura et al., 2005). Two other studies that aimed to evaluate potential laying areas found a median size of 59.9 ha (up to 167.5 ha) in Japan in cuckoos that parasitized GRW, bull-headed shrike, *Lanius bucephalus*, and azure-winged magpie, *Cyanopica cyanus* (Nakamura & Miyazawa, 1997) and 430.0 ha (up to 1510 ha) in Hungary in cuckoos that parasitized GRW (Moskát et al., 2019). Large differences between potential laying areas found in individual studies are probably related to the differences in host species and to the design of the studies, as they were conducted in habitats of different structure and/or using different methods (VHF versus GPS telemetry, see Moskát et al., 2019). An extraordinarily large laying area which we found in one female (2622.2 ha) is in line with Moskát et al. (2019) and the radiotelemetry study by Nakamura and Miyazawa (1997), who observed some cuckoos several kilometres from their potential laying areas, possibly searching

for new mating partners or even host nests. This, together with a relatively high number of females for which we assigned only one egg or chick, suggests that some cuckoo females may follow a 'floating' strategy and search for host nests within a relatively large area.

As spatial characteristics of breeding territories in birds generally depend on the timing of arrival and the ability to compete for limited resources (Greenwood & Harvey, 1982), it seems that this does not hold for cuckoo females which intensively parasitize their hosts within largely overlapping laying areas across the whole breeding season. We found 0–100% overlap of laying areas, which is in broad agreement with Nakamura and Miyazawa (1997), who reported considerable overlap of cuckoo female breeding areas and with Vogl et al. (2004), who also used radiotelemetry and found cuckoo neighbouring home ranges at pond edges overlapping by 20–86% with female–female aggression nearly absent at the same study site (see also Moskát, Hauber, Růžičková, Marton, Bán, & Elek, 2020). There is also evidence for largely overlapping laying areas in great spotted cuckoos, *Clamator glandarius* (Bolopo et al., 2017; Martínez et al., 1998), screaming cowbirds, *Molothrus rufoaxillaris*, shiny cowbirds, *M. bonariensis* (Scardamaglia & Reboreda, 2014) and brown-headed cowbirds, *M. ater* (Fleischer, 1985; Rivers, Young, Gonzalez, Horton, Lock, & Fleischer, 2012; Strausberger & Ashley, 2003; but see Alderson et al., 1999). In contrast, Horsfield's bronze-cuckoos, *Chrysococcyx basalis*, parasitized host nests within exclusive breeding areas (Langmore et al., 2007). Vogl et al. (2004) suggested that the cuckoo laying areas are either difficult to defend due to a high abundance of conspecifics and feeding outside the laying areas, or that defence is not necessary due to the large number of host nests (although this is not the case in our heavily parasitized study site). Moreover, as individual cuckoo females spend a lot of time in the vicinity of the nest selected for parasitism (Honza et al., 2002), it would be difficult to defend a broader laying area at the same time. Thus, it seems that cuckoo

females preferentially invest their energy to search for and parasitize host nests rather than to compete with conspecifics (see Moskát et al., 2020; Vogl et al., 2004).

As predicted, the size of cuckoo laying areas was tightly related to the density of GRW nests: low density of host nests led to larger cuckoo female laying areas and vice versa. Langmore et al. (2007) also found evidence that density and distribution of host territories influence the size of breeding ranges in Horsfield's bronze-cuckoos. In contrast, increased proximity to other nests of Eurasian magpies, *Pica pica*, reduced the probability of being parasitized by great spotted cuckoos (Martinez, Soler, & Soler, 1996). Our results did not suggest that shorter distances between GRW nests prevent them from being parasitized. The reason might be the small body size of GRWs and their limited ability to effectively defend their nests against parasitism (Jelínek et al., 2021; but see Šulc et al., 2020b).

In line with our expectation, individual cuckoo females laid their eggs in areas of similar size and position in both study years. Breeding-site fidelity is common in cuckoos (Koleček et al., 2020; see also Chance, 1940; Moskát et al., 2019) and is thus also apparent at a fine scale as in the GRW at the study site (Koleček et al., 2015). A similar size and position of home ranges across 3 years was also documented in the brown-headed cowbird (Hahn et al., 1999).

In total, two of 27 cuckoo females with at least two assigned offspring (7.4%) parasitized two host species. This agrees with previous evidence that brood parasites may show both host-specialist and host-generalist laying strategies (Alderson et al., 1999; de la Colina, Hauber, Strausberger, Reboreda, & Mahler, 2016; Langmore & Kilner, 2007). In cuckoos, Honza et al. (2002) observed in their radiotelemetry study (carried out in the same area as the present study) that two of seven tracked females (28.6%) parasitized two host species (one RW, one MW and six RW, two SW) and Fossøy et al. (2011) found molecular evidence for one of 15 cuckoo females (6.7 %) with offspring in nests of two host species (eight MW, one GRW) in Bulgaria.

16

In contrast, Skjelseth et al. (2004) assigned offspring of a limited sample of three cuckoo females to just a single host species (six RW, two RW, two GRW) using molecular methods at our study site. Similarly, radiotelemetry studies from Japan (Nakamura & Miyazawa, 1997; Nakamura et al., 2005) reported that each cuckoo female specializes in just one host species (N = 22 females). The molecular approach, however, revealed that two of 24 cuckoo females (8.3%) laid eggs in the nests of two host species (GRW, azure-winged magpie and bull-headed shrike, azure-winged magpie) at the same site (Marchetti et al., 1998). These findings suggest that a small proportion of female cuckoos regularly lay eggs in the nests of other than their main host species and the (possibly unwanted) choice of an alternative host may potentially facilitate the evolution of new host-specific races (gentes; Davies 2000). As the proportion of such females is relatively low, studies should investigate a sufficient sample and molecular methods should be used to prevent biased conclusions when studying this rare phenomenon.

When choosing a suitable nest for parasitism, a cuckoo female preferentially parasitized the GRW nest closest to the nest she had previously parasitized. Cuckoo females spend more time near the host nest chosen for parasitism (Honza et al., 2002), and they probably check the condition of other nests within the same part of the study site. However, why in some cases females parasitized more distant host nests when a much closer nest suitable for parasitism was available remains unclear. For instance, some close, but well-concealed nests could have remained undetected, or the female changed her mate and thereby laying area. Another explanation could be that cuckoo females may preferentially parasitize host nests with eggs more similar to their own (Honza, Šulc, Jelínek, Požgayová, & Procházka, 2014; but see Yang, Wang, Liang, & Møller, 2016; 2017) rather than selecting nests based on the distance. Additional factors such as copying of laying behaviour (watching other females to find the nests) observed in other females may also affect the selection of host nests. We are also aware of one case when a cuckoo female that parasitized GRW laid one egg in the nest of RW in the

same year. Although it was at the end of the breeding season (20 June), six GRW nests suitable for parasitism were available in various parts of the study site at the same time. Unfortunately, the last known egg of this female was laid 18 days earlier; thus, we do not know whether the RW nest was markedly closer than the closest GRW nest available. Since the proportion of eggs laid in the nests of alternative host species is similar to the proportion of eggs laid erroneously (i.e. outside the host egg-laying period; Honza et al., 2020), we suggest that the eggs laid in the nests of other than primary hosts at suitable conditions could represent just another case of a laying error.

To summarize, cuckoo parasitism is host specific and the laying areas are highly overlapping and variable in time and space. While we revealed that cuckoo females preferentially parasitize close to previously parasitized nests, additional focus on this topic is needed especially in communities with different densities of hosts and parasites. Therefore, future research should explain how host population density influences the laying behaviour of brood parasites, that is, how it affects fitness of individual parasitic females and their laying strategies. For instance, female cuckoos moving further from a core laying area to parasitize hosts in new sites may need to meet a threshold density of a potential host species. Further hypotheses may test whether particular females (e.g. first-year breeders) are more constrained in their egg laying than other (e.g. older) females, particularly with respect to the activity of their conspecifics and to the breeding of their hosts.

18

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679

680   Appendix

681

682 **Table 1**

683 Number of active nests (i.e. including at least one host egg), parasitized nests, parasitic offspring

684 (eggs and chicks) and cuckoo females that entered the analyses

Species	Active nests	Parasitized nests	Parasitic offspring	Assigned offspring		No. of cuckoo females	
				MA	PA	Trapped	MA
2016							
Great reed warbler	97	57	91	48	13	5	8
Eurasian reed warbler	383	45	46	36	0	3	9
Sedge warbler	6	1	1	1	0	0	1
Marsh warbler	3	1	1	1	0	0	0
Eurasian reed + marsh warbler	–	–	–	–	–	0	1
2017							
Great reed warbler	95	71	135	63	17	5	9
Eurasian reed warbler	576	96	98	57	8	3	16
Sedge warbler	23	0	0	0	0	0	0
Marsh warbler	5	1	1	0	0	0	0
Unknown host species	1	1	1	0	0	0	0
Great + Eurasian reed warbler	–	–	–	–	–	1	0
Total	1 189	273	374	206	38	9	38

685

686 MA = identity inferred from molecular assignment of offspring; PA = identity inferred from

687 phenotypic assignment of eggs. Trapped = no. of mist-netted females with DNA samples. See

688 Methods for details.

Table A1

Number of offspring assigned to individual cuckoo females by molecular analysis (N_{MA}) and

including the eggs assigned by phenotypic determination (N_{All})

Year	Female ID	N_{MA}	N_{All}	Laying area	Area overlap	Host density	First lay date	Host
2016	#01	6	6	72 784	100	–	3 June	RW
2017	#01	4	4	7 839	100	–	16 May	RW
2016	#02	4	7	349 661	42	0.37	8 May	GRW
2017	#02	6	14	751 118	87	0.31	9 May	GRW
2016	#03	1	1	–	–	–	20 May	RW
2016	#04	2	2	–	–	–	25 May	RW
2016	#05	2	2	–	–	–	10 June	GRW
2016	#06	3	3	5 790	0	–	22 May	RW, MW
2016	#07	1	1	–	–	–	28 May	SW
2016	#08	3	3	398 769	19	–	13 May	RW
2017	#08	3	3	18 602	41	–	8 May	RW
2017	#09	9	11	1 399 716	100	–	19 May	RW
2016	#10	1	1	–	–	–	21 May	RW
2016	#11	2	2	–	–	–	20 May	RW
2017	#11	2	2	–	–	–	23 May	RW
2016	#12	5	6	2 219 165	18	0.07	8 May	GRW
2017	#12	3	3	60 160	84	0.50	28 May	GRW
2016	#13	1	1	–	–	–	18 May	RW
2016	#14	1	1	–	–	–	21 June	GRW
2017	#14	6	7	26 221 591	7	0.01	20 May	GRW
2016	#15	1	1	–	–	–	28 May	GRW
2016	#16	1	1	–	–	–	10 May	RW
2016	#17	3	3	108	100	–	25 May	RW
2016	#18	1	1	–	–	–	10 May	GRW
2016	#19	1	1	–	–	–	–	GRW
2016	#20	1	1	–	–	–	–	GRW
2017	#21	1	1	–	–	–	15 June	GRW
2017	#22	3	3	29 452	97	–	22 May	RW
2017	#23	1	1	–	–	–	6 June	RW
2017	#24	5	5	796 082	25	0.10	2 June	GRW
2017	#25	2	2	–	–	–	16 June	GRW
2017	#26	1	1	–	–	–	29 May	RW
2017	#27	1	1	–	–	–	23 June	RW
2017	#28	3	6	82 462	100	–	26 May	RW
2017	#29	1	1	–	–	–	17 May	RW
2017	#30	3	3	7 397	0	–	2 June	RW
2017	#31	1	1	–	–	–	21 May	RW
2017	#32	1	1	–	–	–	20 May	RW
2017	#33	2	2	–	–	–	–	GRW
2017	#34	1	1	–	–	–	–	GRW
2017	#35	1	1	–	–	–	20 May	RW
2017	#36	2	2	–	–	–	7 June	RW

2017	#37	1	1	–	–	–	14 June	GRW
2017	#38	1	1	–	–	–	24 May	RW
2016	#39	7	7	323 121	67	–	21 May	RW
2017	#39	4	4	9 181	100	–	19 May	RW
2016	#40	4	4	111 571	100	0.54	24 May	GRW
2017	#40	11	13	1 871 355	73	0.13	14 May	GRW
2017	#41	5	5	264 546	100	0.45	25 May	GRW
2016	#42	11	14	655 441	51	0.29	10 May	GRW
2017	#42	3	3	63 762	67	0.78	22 May	GRW
2016	#43	7	11	1 502 147	48	0.16	6 May	GRW
2017	#43	10	15	758 036	91	0.29	15 May	GRW
2016	#44	9	11	568 918	100	0.25	10 May	GRW
2017	#44	4	5	36 401	100	1.92	15 May	GRW
2016	#45	4	4	38 357	58	–	24 May	RW
2017	#45	6	8	891 756	93	–	19 May	RW
2016	#46	3	3	8 661	48	–	4 June	RW
2017	#46	9	10	175 363	99	–	29 May	RW
2016	#47	1	1	–	–	–	30 May	GRW
2017	#47	4	4	37 791	99	–	12 May	RW, GRW

Variables entering the analyses: size of laying area (laying area, m^2), overlap of laying area with laying areas of other females (area overlap, %), host density (GRW nests/1 ha), laying date of the first parasitic egg (first lay date) and identity of host species (host: GRW: great reed warbler; MW: marsh warbler; RW: Eurasian reed warbler; SW: sedge warbler). See Methods for details. Identities of females #1–#38 were revealed by molecular analysis and identities of #39–#47 were inferred from mist netting.

Figure legends

Figure 1. Positions of host nests with offspring belonging to individual cuckoo females in 2016 and 2017. Polygons join the nests on the borders of laying areas of females with at least three offspring. Segments join positions of parasitized nests of cuckoo females with only two offspring. Single points outside the respective polygons and segments represent the females with only one offspring. For individual laying areas see https://isobirdnet.shinyapps.io/Cuckoo_territory/.

Figure 2. Number of offspring assigned to individual cuckoo females parasitizing great reed warblers (GRW) and Eurasian reed warblers (RW) in 2016 and 2017 ($N = 58$ including 13 females with offspring assigned in both years). An additional female parasitized a sedge warbler and two females parasitized two hosts (not shown, see Table 1 and Appendix Table A1 for details).