1 **Multiple parasitism in an evictor brood parasite: Patterns revealed by long-term monitoring, continuous**

2 **video-recording and genetic analyses**

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4 Marcel Honza[1,*], Jaroslav Koleček[1,2], Lubomír Piálek[1,3], Radka Piálková[1,3], Milica Požgayová[1], Petr Procházka[1],

5 Gabriela Štětková[1,4], Václav Jelínek[1], Anna E. Hughes[5] and Michal Šulc[1]

6

7 *[1] Czech Academy of Sciences, Institute of Vertebrate Biology, Brno, Czech Republic*

8 *[2] Institute for Environmental Studies, Charles University, Prague, Czech Republic*

9 *[3] Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic*

10 *[4] Department of Ecology, Faculty of Sciences, Charles University, Prague, Czech Republic*

11 *[5] Department of Psychology, University of Essex, Colchester, U.K.*

12

13 **Author for correspondence: honza@ivb.cz*

14

15 M. Honza (https://orcid.org/0000-0003-2441-1376)

16 J. Koleček (https://orcid.org/0000-0003-1069-6593)

17 L. Piálek (https://orcid.org/0000-0003-1881-4646)

18 R. Piálková (https://orcid.org/0000-0002-5134-4474)

19 M. Požgayová (https://orcid.org/0000-0002-3870-3196)

20 P. Procházka (https://orcid.org/0000-0001-9385-4547)

21 G. Štětková (https://orcid.org/0000-0002-4698-6405)

22 V. Jelínek (https://orcid.org/0000-0002-0997-7358)

23 A.E. Hughes (https://orcid.org/0000-0003-2677-1965)

24 M. Šulc (https://orcid.org/0000-0002-7866-1795)

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26

27 **Abstract**

28

29 In some populations of host of brood parasites, more than two parasite eggs may be laid in a single nest. This

30 phenomenon is known as multiple parasitism, representing a cost to both host and parasite. In this study, we

31 analysed a long-term dataset (2007–2021) focusing on multiple parasitism of the common cuckoo (*Cuculus*

32 *canorus*) parasitizing the great reed warbler (*Acrocephalus arundinaceus*). Annual parasitism rate was on

33 average 54.3% and varied between 5.8% and 92.2%, depending on the year. From 720 parasitized nests, double

34 parasitism was recorded in 172 (23.9%) nests, triple in 51 (7.1%) nests, quadruple in 10 (1.3%) nests and,

35 exceptionally, in the years of heavy parasitism (about 90%), quintuple parasitism was recorded in three (0.4%)

36 nests. The rate of multiple parasitism ranged from 0% to 63% inter-annually and strongly correlated with

37 parasitism rate and total number of parasite eggs found. Furthermore, the number of cuckoo eggs laid per one

38 nest increased with decreasing daily availability of host nests that were at a suitable breeding stage for

39 parasitism. Both genetic and egg phenotype analyses revealed that no cuckoo female laid more than one egg in

40 the same host nest. Using data on long-term parasite–host interactions and from continuous video-recording,

41 as well as progressive methods to assign parasite offspring thus helped us better understand various aspects of

42 multiple parasitism in hosts heavily parasitized by an evictor brood parasite.

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44 Keywords: brood parasitism, common cuckoo, coevolution, egg phenotype, great reed warbler

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53 **Author contributions** MH, MŠ, MP, PP, GŠ, VJ and JK contributed to the study conceptualization and field data

54 collection; LP and RP conducted lab work and bioinformatics analyses; JK, AEH and MŠ analysed the data

Data availability The datasets generated and/or analysed during the current study are available from the supplementary information file.

Ethics approval The fieldwork complied with the current laws and ethical guidelines of the Czech Republic (permit numbers 483/2005-PA, JMK 20189/2010, JMK 115874/2013 and JMK 38506/2016). The use of animals adheres to the guidelines set forth by the Animal Behavior Society/Association for the Study of Animal Behaviour.

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Conflict of interest The authors declare no competing interests.

Significance statement Laying more parasite eggs in one host nest (i.e. multiple parasitism) is common in the brood parasites whose nestlings share the nest with nest mates. In the species where the parasite nestling kills its nest mates, multiple parasitism should be rare because it is costly for the parasite. However, in host populations with high parasitism rates, multiple parasitism occurs more often than predicted. Using long-term and video-recording data, we quantified multiple parasitism in the common cuckoo across years and host egg-laying sequence. We found that the rate of multiple parasitism is positively related to parasitism rate and that the lower the number of nests suitable for parasitism, the higher the number of parasite eggs in one nest.

83   Based on genetic and egg phenotype analyses, we also showed that individual parasitic females avoid laying in

84   the nests they had already parasitized.

85

Introduction

Most birds reproduce by laying eggs in their own nests, yet, about 1% of species are exceptions. These, called interspecific brood parasites, such as many cuckoos (Cuculidae) and cowbirds (Icteridae), lay eggs in the nests of other species (hosts), which then care for the parasite offspring (Davies 2000). To lay their eggs successfully, adult brood parasites have evolved a number of adaptations, namely nest searching behaviour (Kattan 1997; Honza et al. 2002), egg laying mode (Moksnes et al. 2000; Jelínek et al. 2021), and synchronization with host reproduction (Moskát et al. 2006; Geltsch et al. 2016; Honza et al. 2020). Though it might seem that these attributes are well suited to a parasitic lifestyle, not all studies support this. For example, utilization of cavity-nesting species whose nest design prevents young parasites from fledging, or host species that feed their young with diet unsuitable for the parasite (Moksnes and Røskaft 1995; Yang et al. 2013; Liu et al. 2019) could be considered as errors in choosing the appropriate host. Even in the case of correct host selection, laying in abandoned or already incubated nests may decrease the fitness of the parasite (Geltsch et al. 2016; Honza et al. 2020). Another fitness cost for the parasite may arise when one host nest contains several parasite eggs, a phenomenon known as multiple parasitism or multiparasitism.

Rothstein (1990) supposed that multiple parasitism only occurs regularly in parasite–host systems in which the parasite nestling does not actively kill host eggs or young but shares the nest with them at least for a certain period of time (so called non-evictor parasites). Accordingly, multiple parasitism is typical of the brown-headed cowbird, *Molothrus ater* (Trine 2000; Hoover 2003; McLaren et al. 2003), the shiny cowbird, *M. bonariensis* (Lea and Kattan 1998; Tuero et al. 2007; de la Colina et al. 2020), the bronzed cowbird, *M. aeneus* (Ellison et al. 2006) and the screaming cowbird, *M. rufoaxillaris* (De Mársico et al. 2013; De Mársico and Reboreda 2014), as well as of the great spotted cuckoo, *Clamator glandarius* (Soler et al. 1994; Martínez et al. 1998a). Yet, systematic research over the last two decades has shown that in some areas multiple parasitism is frequent even in parasites whose young remove all eggs and/or nest mates shortly after hatching (so called evictor parasites; Moskát and Honza 2002; Spottiswoode 2013; Gloag et al. 2014a; Zölei et al. 2015).

To determine whether multiple parasitism represents a loss or gain for the parasite is not yet known as the results published so far are inconsistent. In non-evictor parasites, multiple parasitism can result in severe competition among parasite nestlings and thus is generally assumed to be costly. In this vein, Goguen et al. (2011) reported that multiple parasitism in the brown-headed cowbird led to lower parasite fledging success

compared to single parasitism. On the other hand, Martínez et al. (1998b) argued that multiple parasitism is not costly for the great spotted cuckoo because its magpie (*Pica pica*) hosts are able to successfully rear several parasite nestlings (see also Soler et al. 1998). In this system, multiple parasitism may even be beneficial for the parasite nestlings once they leave the nest because they join together and are attended by groups of hosts (Soler et al. 2015). Compared to the non-evictor parasites, the cost of multiple parasitism in evictor parasites may be greater because only one parasite nestling can fledge from the multiply parasitized nest. While it seems that this should strongly reduce the fitness of the parasitic females, paradoxically, in some areas the reverse is true. For instance, in areas of heavy parasitism by the common cuckoo (*Cuculus canorus*; hereafter cuckoo), multiple parasitism of the great reed warbler (*Acrocephalus arundinaceus*) nests resulted in decreased rejection of cuckoo eggs, leading to higher cuckoo fledging success (Moskát et al. 2009; Manna et al. 2019).

The cuckoo is an obligate brood parasite widespread over the Palearctic where it regularly parasitizes more than 100 passerine species (Moksnes and Røskaft 1995). Normally, only one cuckoo egg appears in most parasitized nests (Moksnes and Røskaft 1995). However, in areas of high parasitism rate and lack of suitable host nests, the competition among individual cuckoo females is severe and, thus, the incidence of multiple parasitism is also high (Moskát and Honza 2002). This is recently the case in some central European localities, such as the Hungarian Great Plain, where the great reed warblers experience unusually high levels of parasitism (64%). Such parasitism rates can lead to frequent occurrence of multiple parasitism, with 36% of the parasitized nests containing more than one cuckoo egg (Moskát and Honza 2002). In an extreme case, even up to five cuckoo eggs have been found in one nest (Marton 2021).

It has long been supposed that multiple parasitism in the cuckoo is a result of several females parasitizing the same host nests, because if it originated from the same females, it would be maladaptive (Chance 1940; Wyllie 1981; Brooker and Brooker 1990). This suggestion was based on the fact that cuckoo females form host-specific races with characteristic egg appearance (Gibbs et al. 2000; Šulc et al. 2021) and that individual females lay eggs of different appearance (Moksnes et al. 2008). However, the appearance of cuckoo eggs was assessed by humans and only recently it was shown that this method is far from being reliable (Šulc et al. 2021). Therefore, with no genetic support, it is difficult to determine the origin of parasite eggs in multiply parasitized nests.

In this study, we explored multiple parasitism in another great reed warbler population that is under heavy cuckoo parasitism (>50%) in the last decade. Our long-term dataset (15 years) of parasite–host

144 interactions allowed us to explore the temporal patterns of multiple parasitism as well as to test the main

145 factors affecting the incidence of this phenomenon. Moreover, we made use of the data collected for the

146 studies of Koleček et al. (2021) and Šulc et al. (2021) and applied the objective methods of egg assignment to

147 explore how many cuckoo females are involved in multiple parasitism. This allowed us to test the hypothesis

148 that individual cuckoo females do not lay more than one egg per host nest.

149

150 **Methods**

151

152 *Fieldwork and data collection*

153 The study was carried out in the fishponds near Mutěnice (48°54'N, 17°02'E) and Hodonín (48°51'N, 17°07'E) in

154 the Czech Republic from May to July 2007–2021 on a colour-ringed great reed warbler population consisting of

155 40–120 breeding pairs (depending on year). Upon arrival of great reed warbler males, we mapped their

156 territories nearly on a daily basis and thus we found annually almost all nests including the replacement ones.

157 Most nests were found during building or early egg-laying and only a minority at later stages. To record the

158 laying date of cuckoo eggs, we checked the nests almost every day until host clutch completion. Afterwards,

159 the parasitized nests were visited daily for five days to infer host reaction; then it was less often (typically every

160 3–4 days) until the end of the breeding attempt. The non-parasitized nests were also checked less frequently

161 (for details on nest checks see Honza et al. 2020).

162   In 2016 and 2018–2020 we continuously filmed the majority of great reed warbler nests during the

163 pre- and egg-laying stages to record all parasitism events. The recording set-ups comprised either a rear-view

164 camera connected to a miniature digital video-recorder (both placed in a water-resistant box), or a custom HD

165 camera with an inbuilt recorder. Both set-up types were powered by gel batteries. The batteries were placed

166 far from nests (on the bank) and the boxes with the video-recorders were hung on reeds 2–5 m from the nests

167 and were masked by cloths. Cameras were placed on thin aluminium poles camouflaged by cut reed stems and

168 leaves. The distance between the camera and the nest varied from 0.2 to 2 m, depending on reed density. The

169 set-up was mostly installed a day before the first great reed warbler egg was laid. The filmed nests were visited

170 daily to check the nest content and download the data (for technical details about video-recording see Jelínek

171 et al. 2021). The final dataset of filmed nests comprised 242 parasitism events at 151 great reed warbler nests.

172   In 220 filmed events, we knew the exact dates of parasitism (i.e. laying dates of cuckoo eggs) as well as the

173   phase of the host egg laying cycle when the nest was parasitized.

174   In 2016 and 2017, we collected the data for egg-phenotype and genetic analyses. When a cuckoo egg

175   was found, we measured its background colour spectrophotometrically (taking nine measurements from three

176   egg parts). At the same time, we took a digital photograph of each egg. The photos were taken in RAW format,

177   under diffuse sunlight conditions, at the same angle and from the same distance and were referred to a grey

178   standard (for technical details about egg-phenotype measurement see Šulc et al. 2021).

179   DNA samples of cuckoo offspring in these two years were collected as follows: since only one cuckoo

180   nestling usually survives in parasitized nests, we removed the newly laid (second and following) cuckoo eggs

181   from multiply parasitized nests shortly after laying (i.e. we left only the first laid cuckoo egg). As DNA from the

182   freshly laid eggs could not be effectively sampled, we transferred the removed eggs to an incubator (HEKA-

183   Kongo; HEKA-Brutgeräte, Rietberg, Germany) and incubated them artificially until hatching. Fresh hatchlings

184   were then moved to suitable host nests for other studies. When the nestling achieved the age of about 10

185   days, we took a blood sample (approx. 25 µl) from its ulnar or medial tarsometatarsal vein. In the case of

186   unhatched or dead nestlings, we used tissue samples instead. In 2016 and 2017, we also mist-netted 19 adult

187   cuckoo females and collected their blood samples (as in nestlings). All DNA samples were stored in 96% ethanol

188   until later genetic analyses.

189

190   *Assignment of cuckoo mothers – genetic determination*

191   DNA was extracted from blood or tissue samples using Tissue Genomic DNA mini kit (Geneaid Biotech Ltd, New

192   Taipei, Taiwan). To infer the identity of cuckoo mothers, we used single nucleotide polymorphism (SNP)

193   markers and mitochondrial DNA (see also Koleček et al. 2021; Šulc et al. 2021).

194   Firstly, we genotyped all samples collected in 2016 and 2017 with the ddRAD (double digest

195   restriction-site associated DNA) technique (Peterson et al. 2012) following the protocol of Piálek et al. (2019) to

196   acquire the SNP dataset and determine individual identity. The sample libraries were sequenced on an Illumina

197   HiSeq4000 system (2 lanes, 150 cycles P/E) in the European Molecular Biology Laboratory Genomic Core

198   Facility, Heidelberg, Germany. The obtained RAD-tags were processed in Stacks v2.4 (Catchen et al. 2011;

199   Rochette et al. 2019) and mapped on the cuckoo genome GCA000709325.1 (https://www.ncbi.nlm.nih.gov)

200   with Bowtie2 assembler v2.2.4 (Langmead and Salzberg 2012). Only loci with 95% or higher presence of

201   individuals were scored. The obtained loci were further filtered in PLINK v1.9 (Purcell et al. 2007) so that only

202   the loci in Hardy–Weinberg equilibrium that do not show evidence of linkage disequilibrium and with alleles

203   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

204   dataset with 1620 variants.

205   Secondly, for the mitochondrial haplotype analysis, we sequenced a 411-bp portion of the left-hand

206   hypervariable control region (Fossøy et al. 2011, 2012; Gibbs et al. 2000). Mitochondrial sequence data were

207   assembled and manually checked in Geneious v10.2.6 (Kearse et al. 2012) and haplotypes were estimated

208   based on a distance matrix with up to 1% tolerance (approx. 4 mutations) for genotyping errors.

209   Finally, we determined the identity of cuckoo mothers for individual offspring using the program

210   Colony (Jones and Wang 2010). Colony enables identification of individual offspring and determines their half-

211   and full-sibling relationships using a full-pedigree likelihood approach and also allows the inclusion of additional

212   information about known relationships among the offspring to increase the probability of correct assignment

213   (i.e. based on mtDNA in our case).

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

215   haplotypes enabling to exclude highly implausible maternal (or maternal-sibling) relationships in the inferred

216   genealogy. None of the eggs assigned to a cuckoo female were laid on the same or subsequent day, which

217   agrees with cuckoo laying intervals of around 48 hours (Seel 1973; Wyllie 1975; Nakamura et al. 2005).

218   Using genetic methods, we assigned offspring to individual cuckoo mothers in 27 multiply parasitized

219   nests. In additional 29 multiply parasitized nests, however, we assigned no or only one offspring due to

220   predation or egg ejection by the hosts. For a subset of these 29 nests, we thus used assignment based on egg

221   phenotype (see below).

222

223   *Assignment of cuckoo mothers – egg phenotype determination*

224   Recent studies showed that maternal identity can be encoded in egg phenotype (Gómez et al. 2021; Šulc et al.

225   2021; Johnson and McRae 2022). Therefore, we expanded our dataset of genetically assigned offspring from

226   2016 and 2017 by eggs whose phenotypes were measured but could not be genetically assigned to cuckoo

227   females because no DNA was sampled due to early host ejection or predation. For this purpose, we employed

228   an automatic analytical approach predicting the identity of cuckoo females based on the appearance of their

229   eggs. We evaluated the visual similarities of particular cuckoo eggs in their colour, pattern and egg dimensions

9

from calibrated photographs and spectrophotometry (for details see Šulc et al. 2019). Spectral data of egg

background colour were obtained using spectrophotometry measurements and the R package *pavo* (Maia et al.

2019). Egg dimension data were obtained from the photographs and included the length, maximum width,

volume, ellipse deviation and surface area. Pattern data used custom scripts to calculate pattern energies and

skew metrics that gave measures of how patterned the eggs are and what is the spatial distribution of the

patterns. Finally, luminance data were analysed using the photographs, including both the spots and

background areas of the eggs. All data from digital photographs were obtained by using the Mica Toolbox

(Troscianko and Stevens 2015; van den Berg et al. 2020). In the next step, we trained a random forest model to

automatically assign eggs of the unknown origin to the eggs with known cuckoo mothers. This method showed

97.5% accuracy and assignments did not violate our non-phenotypic criteria, such as (i) two cuckoo eggs of one

female could not be laid on the same or subsequent days and (ii) the cuckoo female had to be the same colour

morph (rufous or grey; data from video-recordings). For more information about the assignment of cuckoo

mothers by egg phenotypes see Šulc et al. (2021). Using this method, we assigned cuckoo offspring in

additional 9 multiply parasitized nests.

Number of cuckoo mothers assigned

In total, we assigned 134 cuckoo eggs to 17 females (12 and 13 in 2016 and 2017, respectively; 8 females were

the same in both years). Out of these 134 eggs, 104 were assigned by using genetic methods and 30 by using

egg phenotype. Assigned cuckoo eggs were laid in 85 great reed warbler nests (41 and 44 in 2016 and 2017,

respectively). Out of these 85 nests, 49 contained one assigned egg and 36 were multiply parasitized.

Data analysis

For each day a cuckoo egg was laid, we calculated the number of host nests available for parasitism. The nest

was available for parasitism from the host clutch initiation date to day 4 of the host egg laying period (or to an

earlier day in case of deserted or predated nests). After day 4, host incubation starts and the probability of

early cuckoo hatching decreases (Geltsch et al. 2016). Host nest availability for a given cuckoo egg-laying date

was then expressed as the sum of the nests available for parasitism on that day (Jelínek et al. 2013). As the

total number of cuckoo eggs found each year in great reed warbler nests may indicate the number of cuckoo

females in the study area, we used this variable as an estimate of cuckoo density.

259   Using the data from continuous video-recording, we tested the effect of daily availability of great reed

260   warbler nests, cuckoo egg laying date and study year (explanatory variables) on the number of cuckoo eggs

261   present in one nest (response variable) using linear mixed-effects models with the R package 'lme4' (Bates et

262   al. 2015). To explore whether the effect of daily availability of great reed warbler nests varied with the egg

263   laying date, we added a two-way interaction between the daily availability of great reed warbler nests and

264   cuckoo egg laying date. The random effects structure contained the great reed warbler nest identity to account

265   for the nests containing multiple cuckoo eggs. To obtain comparable model parameter estimates (Schielzeth

266   2010), we standardized the explanatory variables using the 'scale' function, so that the mean was zero and the

267   standard deviation 1. Model residuals did not indicate any violation of model assumptions.

268   To test whether cuckoo females lay preferentially in different nests, we used a randomization test

269   (Manly 1997). Here we used the dataset of cuckoo eggs with known cuckoo mothers (based on the genetics

270   and egg-phenotype) that were found in multiply parasitized nests (at least two in every nest) and simulated the

271   situation when cuckoo females lay their eggs in the nests randomly. During the procedure, we randomly

272   assigned the respective number of cuckoo eggs (from two to four) to these nests (N = 36, see results) and

273   summed cases when eggs in the same nest were laid by the same cuckoo female(s). This procedure was

274   repeated 9999 times and all 9999 sums were sorted by their values. The number of nests which were naturally

275   parasitized by the same female in our dataset was also included. Finally, we calculated the proportion of all

276   simulated sums that were lower than the number of observed nests parasitized by the same cuckoo female

277   which represents the significance level of the randomization (Manly 1997). All statistical analyses were

278   conducted in R 3.4.1 (R Development Core Team 2017). To minimize observer bias, blinded methods were used

279   when the behavioural data were analyzed.

280

281   **Results**

282

283   *Multiple parasitism across years*

284   In the course of 15 years, cuckoo parasitism rate varied inter-annually from 5.8% (in 2008) to 92.2% (in 2017;

285   Fig. 1A), reaching the average of 54.3%. The annual rate of multiple parasitism ranged from 0 to 63%, and

286   strongly correlated with annual parasitism rate (Pearson's correlation coefficient r = 0.88, P < 0.001; Fig. 2A)

287   and total number of cuckoo eggs found each year (r = 0.86, P < 0.001; Fig. 2B). From a total of 720 parasitized

288 nests, double parasitism was recorded in 172 (23.9%) nests, triple in 51 (7.1%) nests, and quadruple in 10

289 (1.3%) nests. In the years of extremely high parasitism rates (2017 and 2018), quintuple parasitism was

290 recorded in three (0.4%) nests (Fig. 1B).

291

292 *Multiple parasitism during host egg-laying sequence*

293 In years 2016 and 2018–2020, we video-recorded 220 parasitism events in which we were able to determine

294 exact cuckoo egg laying date and egg order. The median laying date of the first cuckoo egg (N = 123) was the

295 same as the host clutch initiation date, the median laying dates of the second (N = 70) and third cuckoo eggs (N

296 = 24) were the day after host clutch initiation, and the median date when the fourth cuckoo egg (N = 3) was laid

297 was one day later. Interestingly, 15 (21.4%) of 70 multiply parasitized nests were at least double parasitized

298 prior to the initiation of host egg laying (Fig. 3).

299

300 *Main factors affecting multiple parasitism*

301 The number of cuckoo eggs in one nest increased with decreasing daily availability of host nests suitable for

302 parasitism (estimate = −0.136, SE = 0.049, χ^2 = 7.58, P = 0.006) and this relationship did not change with the

303 laying date of a cuckoo egg (interaction term: estimate = 0.001, SE = 0.069, χ^2 < 0.01, P = 0.986), determined

304 from the video-recordings. Simultaneously, the number of cuckoo eggs in host nests did not differ with the

305 laying date (estimate = −0.006, SE = 0.049, χ^2 < 0.02, P = 0.902) and study year (estimate = −0.025, SE = 0.049, χ^2

306 = 0.25, P = 0.618).

307

308 *How many cuckoo females are involved in multiple parasitism?*

309 Out of the nests sampled in 2016 and 2017 for egg assignment to individual cuckoo mothers, 36 were multiply

310 parasitized (24 with two cuckoo eggs, 11 with three cuckoo eggs and 1 with four cuckoo eggs). These 36 nests

311 were used by 15 cuckoo females and no cuckoo female laid more than one egg in the same nest. A

312 randomization test showed that this observation significantly differed from chance (P < 0.001). The same result

313 was obtained when we used a reduced dataset consisting only of genetically assigned cuckoo eggs (N = 57 eggs

314 laid in 27 nests by 14 cuckoo females, P = 0.008).

315

316 **Discussion**

317

Patterns of multiple parasitism

The main assumption for the presence of multiple parasitism in a host population is that cuckoo females are not strictly territorial and their laying ranges overlap to a large extent (Nakamura and Miyazawa 1997; Koleček et al. 2021). Likewise, the studies on great spotted cuckoos and brown-headed cowbirds indicate that multiple parasitism may be a consequence of the non-territorial behaviour of parasitic females (Martínez et al. 1998b; de la Colina et al. 2016). We found that in our cuckoo–great reed warbler system, the rate of multiple parasitism strongly correlates with the parasitism rate of host nests and, most probably, depends on two main population parameters: parasite and host densities. Similarly, Yang et al. (2014) reported that the rates of multiple parasitism were positively correlated with local cuckoo parasitism rate in the Oriental reed warbler (*Acrocephalus orientalis*). This scenario might apply also to central Hungary where the great reed warbler hosts are under relatively high cuckoo parasitism (about 64%) and similarly intensive multiple parasitism (Moskát and Honza 2002; Zölei et al. 2015) as we found. Interestingly, in 2017 and 2018 when the parasitism rate at our study site reached about 90%, we found three host nests parasitized with five cuckoo eggs. A similar but single case of a quintuple parasitism of a great reed warbler nest was recently recorded in central Hungary (Marton et al. 2021). On the other hand, the host populations exhibiting low levels of parasitism (up to 30%) typically do not have their nests multiply parasitized or only very rarely (Moksnes et al. 1993; Rutila et al. 2002; Antonov et al. 2006; our unpublished data on a sympatric Eurasian reed warbler, *Acrocephalus scirpaceus*, population). This also seems to be the case of our data, as in 2008 the parasitism rate dropped to 6% and that year was the only year in which we have not recorded any case of multiple parasitism over a 15-year study period.

Our results further demonstrated that multiple parasitism increases with decreasing number of available host nests that are at a suitable breeding stage for parasitism. In addition, though we do not have exact information on the number of cuckoo females operating in our study area, as a proxy of this we used total number of cuckoo eggs found each year and showed that the latter strongly correlates with the prevalence of multiple parasitism. It is therefore possible that the locally high density of cuckoo females contributes to the extraordinarily (at least in some years) high proportion of multiple parasitism. Moreover, individual parasitic females may eavesdrop on alarm or mobbing calls of their hosts (Marton et al. 2019) or on behaviour of other parasitic females (Gloag et al. 2013) and, thus, multiple females may parasitize the same host nests. It is known that the great reed warbler is one of the most aggressive cuckoo hosts (e.g. Trnka and

346 Prokop 2012; Požgayová et al. 2013); however, as it has recently been shown, its aggressiveness does not

347 protect it from parasitism (Jelínek et al. 2021). On the contrary, the high host aggressiveness may serve as a cue

348 for the parasitic female and thus contribute to the high incidence of multiple parasitism.

349   Finally, our results revealed that the majority of multiple parasitism events occurred during host egg

350 laying. Interestingly, a considerable proportion of cuckoo females (21%) made a mistake and laid their eggs

351 prior to the initiation of host clutch, which is five times more often compared to single parasitism (Honza et al.

352 2020). This finding supports the previous assumption that the lack of suitable host nests and high cuckoo

353 density lead to high rate of multiple parasitism and also to a high rate of laying errors.

354

355 *How do the females avoid multiple parasitism of the same nests?*

356 Previous studies based on human assessment of cuckoo eggs suggested that the eggs in multiply parasitized

357 nests belonged to different parasitic females (Moksnes et al. 2008). Our study supported this assumption

358 genetically and by using objective egg assessment methods. The reproductive strategy of avoiding multiple

359 parasitism of the same nests by single females has also been reported in the bronzed cowbird (Ellison et al.

360 2006) and screaming cowbird (Ursino et al. 2020). In the brown-headed and shiny cowbirds, however, there is a

361 mix of two multiple parasitism strategies. In some populations, individual parasitic females avoid parasitizing

362 the nests they have already parasitized (see Ellison et al. 2006; Gloag et al. 2014b for the two species,

363 respectively), while in others about one half of the multiply parasitized nests contained eggs laid by the same

364 females (McLaren et al. 2003; de la Colina et al. 2016, respectively). It has been suggested that the ability to

365 remember the nests that a female has already parasitized is facilitated by a larger hippocampus and smaller

366 home ranges in females compared to males (Reboreda et al. 1996). The lack of information on the

367 hippocampus size in cuckoos (Reboreda et al. 1996) therefore makes the question about its importance in

368 avoiding multiple parasitism by the same female even more interesting. Honza et al. (2014) showed that

369 cuckoo females select certain host nests based on egg appearance to increase the mimicry of their eggs, but

370 cannot recognize the egg of another cuckoo female, or at least do not preferentially dispose of it (Šulc et al.

371 2016). This is an indication that the cognitive abilities of cuckoos to recognize already parasitized nests can be

372 at a lower level.

373

374 *Factors contributing to annual variation in multiple parasitism*

375   We are convinced that several conditions must be met for multiple parasitism to occur in a host population.

376   Our results indicate that multiple parasitism is most probably a consequence of a large concentration of

377   parasites and perhaps the associated decrease in the size of host population which we have observed in our

378   study plot in last years (Fig. 1A). One could expect that multiple parasitism is more costly for evicting brood

379   parasites (cuckoos) than for the parasites whose young develop together with those of their hosts (cowbirds).

380   In cuckoos, from the perspective of an individual, multiple parasitism is clearly costly, because only one cuckoo

381   nestling can fledge from the nest with multiple cuckoo eggs (Wyllie 1981). To reduce such a fitness loss,

382   individual females may then adopt "make the best of a bad job" strategy and lay in nests of an alternative host.

383   This is, however, unlikely in cuckoos because individual parasitic females are almost exclusively host-specific, as

384   was shown at our study site (Honza et al. 2002; Koleček et al. 2021). On the contrary, from the perspective of a

385   parasite population, multiple parasitism may not have so serious impact. Moskát et al. (2009) showed that

386   multiple parasitism caused significant reduction of rejection rates of parasite eggs in the great reed warbler. It

387   was because the hosts were more prone to make recognition errors both when they had a greater proportion

388   of parasite eggs in their clutch and/or when these eggs were more variable in appearance (Manna et al. 2019).

389   The increased host tolerance towards multiple cuckoo eggs might thus result in more than doubled cuckoo

390   reproductive output found in multiply parasitized nests compared to singly parasitized nests (Moskát et al.

391   2009). The authors of this study explained this by the fact that having more than one cuckoo egg per host

392   clutch increased the chance that a cuckoo egg would be accepted and eventually hatch. Thus, a greater

393   proportion of multiply parasitized nests contained a cuckoo hatchling than did singly parasitized nests (Moskát

394   et al. 2009). In another host–parasite system, multiple parasitism is even assumed to be responsible for the

395   evolution of parasite nestling recognition by hosts instead of parasite egg recognition (Sato et al. 2010). One

396   way or another, in agreement with Moskát et al. (2009), we assume that another factor involved in the

397   maintenance of multiple parasitism is the equilibrium between the above-mentioned costs and benefits of

398   cuckoo reproduction.

399   To conclude, our study contributes to the understanding of parasite–host interactions. We quantified

400   some of the factors potentially influencing the incidence of multiple parasitism in a cuckoo host. Moreover, we

401   showed that individual cuckoo females avoid laying again in the nests they had already parasitized. Exciting

402   areas for future research would be to study the cognitive abilities of individual parasitic females associated

403   with the location of host nests in space and time, and to explore the role of eavesdropping by the second-to-

404   parasitize females.

405

406   **References**

407

408   Antonov A, Stokke BG, Moksnes A, Røskaft E (2006) Egg rejection in marsh warblers (*Acrocephalus palustris*)

409   heavily parasitized by common cuckoos (*Cuculus canorus*). Auk 123:419–430.

410   https://doi.org/10.1093/auk/123.2.419

411

412   Barabás L, Gilicze B, Takasu F, Moskát C (2004) Survival and anti-parasite defense in a host metapopulation

413   under heavy brood parasitism: a source–sink dynamic model. J Ethol 22:143–151.

414   https://doi.org/10.1007/s10164-003-0114-y

415

416   Bates D, Mächler M, Bolker BM, Walker SC (2015) Fitting linear mixed-effects models using lme4. J Stat Softw

417   67:1–48. https://doi.org/10.18637/jss.v067.i01

418

419   Brooker LC, Brooker MG (1990) Why are cuckoos host specific? Oikos 57:301–309

420

421   Catchen JM, Amores A, Hohenlohe P, Cresko W, Postlethwait JH (2011) Stacks: Building and genotyping loci de

422   novo from short-read sequences. G3-Genes Genom Genet 1:171–182. https://doi.org/10.1534/g3.111.000240

423

424   Chance EP (1940) The truth about the cuckoo. Country Life, London

425

426   Davies NB (2000) Cuckoos, cowbirds and other cheats. T. & A. D. Poyser, London

427

428   de la Colina MA, Hauber ME, Strausberger BM, Reboreda JC, Mahler B (2016) Molecular tracking of individual

429   host use in the Shiny Cowbird – a generalist brood parasite. Ecol Evol 6:4684–4696.

430   https://doi.org/10.1002/ece3.2234

431

432 De Mársico MC, Gloag R, Ursino CA, Reboreda JC (2013) A novel method of rejection of brood parasitic eggs

433 reduces parasitism intensity in a cowbird host. Biol Lett 9:20130076. http://doi.org/10.1098/rsbl.2013.0076

434

435 De Mársico MC, Reboreda JC (2014) High frequency but low impact of brood parasitism by the specialist

436 screaming cowbird on its primary host, the baywing. Emu 114:309–316. https://doi.org/10.1071/MU14008

437

438 Ellison K, Sealy SG, Gibbs HL (2006) Genetic elucidation of host use by individual sympatric bronzed cowbirds

439 (*Molothrus aeneus*) and brown-headed cowbirds (*M. ater*). Can J Zool 84:1269–1280.

440 https://doi.org/10.1139/z06-091

441

442 Fossøy F, Antonov A, Moksnes A, Røskaft E, Vikan JR. Møller AP, Shykoff JA, Stokke BG (2011) Genetic

443 differentiation among sympatric cuckoo host races: Males matter. Proc R Soc B 278:1639–1645.

444 https://doi.org/10.1098/rspb.2010.2090

445

446 Fossøy F, Moksnes A, Røskaft E, Antonov A, Dyrcz A, Moskát C, Ranke PS, Rutila J, Vikan JR, Stokke BG (2012)

447 Sex allocation in relation to host races in the brood-parasitic common cuckoo (*Cuculus canorus*). PLoS ONE

448 7:e36884. https://doi.org/10.1371/journal.pone.0036884

449

450 Gärtner K (1981) Das Wegnehmen von Wirtsvogeleiern durch den Kuckuck (*Cuculus canorus*). Ornithol Mitt

451 33:115–131

452

453 Geltsch N, Bán M, Hauber ME, Moskát C (2016) When should common cuckoos *Cuculus canorus* lay their eggs

454 in host nests? Bird Study 63:46–51. https://doi.org/10.1080/00063657.2015.1125851

455

456 Gibbs HL, Sorenson MD, Marchetti K, Brooke M de L, Davies NB, Nakamura H (2000) Genetic evidence for

457 female host-specific races of the common cuckoo. Nature 407:183. https://doi.org/10.1038/35025058

458

459 Gloag R, Fiorini VD, Reboreda JC, Kacelnik A (2013) The wages of violence: mobbing by mockingbirds as a

460 frontline defence against brood parasitic cowbirds. Anim Behav 86:1023–1029.

461 https://doi.org/10.1016/j.anbehav.2013.09.007

462

463 Gloag R, Fiorini VD, Reboreda JC, Kacelnik A (2014b) Shiny cowbirds share foster mothers but not true mothers

464 in multiply parasitized mockingbird nests. Behav Ecol Sociobiol 68:681–689. https://doi.org/10.1007/s00265-

465 014-1682-2

466

467 Gloag R, Keller LA, Langmore NE (2014a) Cryptic cuckoo eggs hide from competing cuckoos. Proc R Soc B

468 281:20141014. https://doi.org/10.1098/rspb.2014.1014

469

470 Goguen CB, Curson DR, Mathews NE (2011) Costs of multiple parasitism for an avian brood parasite, the

471 brown-headed cowbird (*Molothrus ater*). Can J Zool 89:1237–1248. https://doi.org/10.1139/Z11-104

472

473 Gómez J, Gordo O, Minias P (2021) Egg recognition: The importance of quantifying multiple repeatable features

474 as visual identity signals. PLoS ONE 16:e0248021. https://doi.org/10.1371/journal.pone.0248021

475

476 Honza M, Požgayová M, Procházka P, Koleček J (2020) Errors in egg laying by female common cuckoo *Cuculus*

477 *canorus* in nests of its common host. Ibis 162:637–644. https://doi.org/10.1111/ibi.12808

478

479 Honza M, Šulc M, Jelínek V, Požgayová M, Procházka P (2014) Brood parasites lay eggs matching the

480 appearance of host clutches. Proc R Soc B 281:20132665. https://doi.org/10.1098/rspb.2013.2665

481

482 Honza M, Taborsky B, Taborsky M, Teuschl Y, Vogl W, Moksnes A, Røskaft E (2002) Behaviour of female

483 common cuckoos, *Cuculus canorus*, in the vicinity of host nests before and during egg laying: a radiotelemetry

484 study. Anim Behav 64:861–868. https://doi.org/10.1093/beheco/arw085

485

486 Hoover JP (2003) Multiple effects of brood parasitism reduce the reproductive success of prothonotary

487 warblers, *Protonotaria citrea*. Anim Behav 65:923–934. https://doi.org/10.1006/anbe.2003.2155

488

489 Jelínek V, Procházka P, Požgayová M, Honza M (2013) Common cuckoos *Cuculus canorus* change their nest-

490 searching strategy according to the number of available host nests. Ibis 156:189–197.

491 https://doi.org/10.1111/ibi.12093

492

493 Jelínek V, Šulc M, Štětková G, Honza M (2021) Fast and furious: host aggression modulates behaviour of brood

494 parasites. Ibis 163:824–833. https://doi.org/10.1111/ibi.12930

495

496 Johnson EW, McRae SB (2022) Interclutch variability in egg characteristics in two species of rail: Is maternal

497 identity encoded in eggshell patterns? PLoS ONE 17:e0261868. https://doi.org/10.1371/journal.pone.0261868

498

499 Jones OR, Wang J (2010) Colony: A program for parentage and sibship inference from multilocus genotype

500 data. Mol Ecol Resour 10:551–555. https://doi.org/10.1111/j.1755-0998.2009.02787.x

501

502 Kattan GH (1997) Shiny cowbirds follow the 'shotgun' strategy of brood parasitism. Anim Behav 53:647–654.

503 https://doi.org/10.1006/anbe.1996.0339

504

505 Kearse M, Moir R, Wilson A et al (2012) Geneious basic: An integrated and extendable desktop software

506 platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

507 https://doi.org/10.1093/bioinformatics/bts199

508

509 Koleček J, Piálková R, Piálek L et al. (2021) Spatiotemporal patterns of egg laying in the common cuckoo. Anim

510 Behav 177:107–116. https://doi.org/10.1016/j.anbehav.2021.04.021

511

512 Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359.

513 https://doi.org/10.1038/nmeth.1923

514

515 Lea SE, Kattan GH (1998) Reanalysis gives further support to the 'shotgun' model of shiny cowbird parasitism of

516 house wren nests. Anim Behav 56:1571–1573. https://doi.org/10.1006/anbe.1998.0925

517

518 Liu J, Yang C, Liang W (2019) Brood parasitism of rosefinches by cuckoos: suitable host or accidental parasitism?

519 J Ethol 37:83–92. https://doi.org/10.1007/s10164-018-0571-y

520

521 Maia R, Gruson H, Endler JA, White TE (2019) Pavo 2: new tools for the spectral and spatial analysis of colour in

522 R. Methods Ecol Evol 10:1097–1107.https://doi.org/10.1111/2041-210X.13174

523

524 Manly BFJ (1997) Monte Carlo methods. In: Randomization, bootstrap, and Monte Carlo methods in biology,

525 2nd edn. Chapman and Hall, London, pp 69–78

526

527 Manna TJ, Moskát C, Tong L, Bán M, Aidala Z, Low J, Hauber ME (2019) Multiple parasitism reduces egg

528 rejection in the host (*Acrocephalus arundinaceus*) of a mimetic avian brood parasite (*Cuculus canorus*). J Comp

529 Psychol 133:351–358. https://doi.org/10.1037/com0000166

530

531 Martínez JG, Burke T, Dawson D, Soler JJ, Soler M, Møller AP (1998a) Microsatellite typing reveals mating

532 patterns in the brood parasitic great spotted cuckoo (*Clamator glandarius*). Mol Ecol 7:289–297.

533 https://doi.org/10.1046/j.1365-294X.1998.00348.x

534

535 Martínez JG, Soler JJ, Soler M, Burke T (1998b) Spatial pattern of egg laying and multiple parasitism in a brood

536 parasite: a non-territorial system in the great spotted cuckoo (*Clamator glandarius*). Oecologia 117:286–294.

537 https://doi.org/10.1007/s004420050660

538

539 Marton A (2021) Quintuple parasitism of a great reed warbler nest by common cuckoos. Ecol Evol 11:8420–

540 8423. https://doi.org/10.1002/ece3.7669

541

542 Marton A, Fülöp A, Ozogány K, Moskát C, Bán M (2019) Host alarm calls attract the unwanted attention of the

543 brood parasitic common cuckoo. Sci Rep 9:18563. https://doi.org/10.1038/s41598-019-54909-1

544

545   McLaren CM, Woolfenden BE, Gibbs HL, Sealy SG (2003) Temporal patterns of multiple parasitism by brown-

546   headed cowbirds (*Molothrus ater*) on song sparrows (*Melospiza melodia*). Can J Zool 81:281–286.

547   https://doi.org/10.1139/z03-002

548

549   Moksnes A, Røskaft E (1995) Egg-morphs and host preference in the common cuckoo (*Cuculus canorus*): an

550   analysis of cuckoo and host eggs from European museum collections. J Zool 236:625–648.

551   https://doi.org/10.1111/j.1469-7998.1995.tb02736.x

552

553   Moksnes A, Røskaft E, Bičík V, Honza M, Øien IJ (1993) Cuckoo *Cuculus canorus* parasitism on *Acrocephalus*

554   warblers in Southern Moravia in the Czech Republic. J Ornithol 134:425–434

555

556   Moksnes A, Røskaft E, Hagen LG, Honza M, Mørk C, Olsen PH (2000) Common cuckoo *Cuculus canorus* and host

557   behaviour at reed warbler *Acrocephalus scirpaceus* nests. Ibis 142:247–258. https://doi.org/10.1111/j.1474-

558   919X.2000.tb04864.x

559

560   Moksnes A, Røskaft E, Rudolfsen G et al (2008) Individual female common cuckoos *Cuculus canorus* lay

561   constant egg types but egg appearance cannot be used to assign eggs to females. J Avian Biol 39:238–241.

562   https://doi.org/10.1111/j.2008.0908-8857.04158.x

563

564   Moskát C (2005). Common Cuckoo parasitism in Europe: Behavioural adaptations, arms race and the role of

565   metapopulations. Ornithol Sci 4:3–15. https://doi.org/10.2326/osj.4.3

566

567   Moskát C, Barta Z, Hauber ME, Honza M (2006) High synchrony of egg laying in common cuckoos (*Cuculus*

568   *canorus*) and their great reed warbler (*Acrocephalus arundinaceus*) hosts. Ethol Ecol Evol 18:159–167.

569   https://doi.org/10.1080/08927014.2006.9522720

570

571   Moskát C, Hauber ME, Avilés JM, Bán M, Hargitai R, Honza M (2009) Increased host tolerance of multiple

572   cuckoo eggs leads to higher fledging success of the brood parasite. Anim Behav 77:1281–1290.

573   https://doi.org/10.1016/j.anbehav.2009.01.030

574

575   Moskát C, Honza M (2000) Effect of nest and nest site characteristics on the risk of cuckoo *Cuculus canoru*s

576   parasitism in the great reed warbler *Acrocephalus arundinaceus*. Ecography 23:335–341.

577   https://doi.org/10.1111/j.1600-0587.2000.tb00289.x

578

579   Moskát C, Honza M (2002) European cuckoo *Cuculus canorus* parasitism and host's rejection behaviour in a

580   heavily parasitized great reed warbler *Acrocephalus arundinaceus* population. Ibis 144:614–622.

581   https://doi.org/10.1046/j.1474-919X.2002.00085.x

582

583   Nakamura H, Miyazawa Y (1997) Movements, space use and social organisation of radiotracked common

584   cuckoos during the breeding season in Japan. Japn J Ornithol 46:23–54. https://doi.org/10.3838/jjo.46.23

585

586   Nakamura H, Miyazawa Y, Kashiwagi K (2005) Behavior of radio-tracked common cuckoo females during the

587   breeding season in Japan. Ornithol Sci 4:31–41. https://doi.org/10.2326/osj.4.31

588

589   Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE (2012) Double digest RADseq: An inexpensive method

590   for de novo SNP discovery and genotyping in model and non-model species. PLoS ONE 7:e37135.

591   https://doi.org/10.1371/journal.pone.0037135

592

593   Piálek L, Burress E, Dragová K, Almirón A, Casciotta J, Říčan O (2019) Phylogenomics of pike cichlids (Cichlidae:

594   Crenicichla) of the *C. mandelburgeri* species complex: Rapid ecological speciation in the Iguazú river and high

595   endemism in the Middle Paraná basin. Hydrobiologia 832:355–375. https://

596   doi.org/10.1007/s10750-018-3733-6

597

598   Požgayová M, Procházka P, Honza M (2013) Is shared male assistance with antiparasitic nest defence costly in

599   the polygynous great reed warbler? Anim Behav 85:615–621. https://doi.org/10.1016/j.anbehav.2012.12.024

600

601   Purcell S, Neale B, Todd-Brown K, Thomas L et al (2007) Plink: A tool set for whole-genome association and

602   population-based linkage analyses. Am J Hum Genet 81:559–575. https://doi.org/10.1086/519795

603

604 R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical

605 Computing, Vienna, https://www.R-project.org/

606

607 Reboreda JC, Clayton NS, Kacelnik A (1996) Species and sex differences in hippocampus size in parasitic and

608 nonparasitic cowbirds. Neuroreport 7:505–508. https://doi.org/10.1097/00001756-199601310-00031

609

610 Rochette N, Rivera-Colón A, Catchen J (2019) Stacks 2: Analytical methods for paired-end sequencing improve

611 RADseq-based population genomics. Mol Ecol 28:4737–4754. https://doi.org/10.1111/mec.15253

612

613 Rothstein SI (1990) A model system for coevolution: avian brood parasitism. Annu Rev Ecol Syst 21:481–508

614

615 Rutila J, Latja R, Koskela K (2002) The common cuckoo *Cuculus canorus* and its cavity nesting hosts, the redstart

616 *Phoenicurus phoenicurus*: a peculiar cuckoo–host system? J Avian Biol 33:414–419.

617 https://doi.org/10.1034/j.1600-048X.2002.02937.x

618

619 Sato NJ, Mikami OK, Ueda K (2010) The egg dilution effect hypothesis: a condition under which parasitic

620 nestling ejection behaviour will evolve. Ornithol Sci 9:115–121. https://doi.org/10.2326/osj.9.115

621

622 Scardamaglia RC, Reboreda JC (2014) Ranging behavior of female and male shiny cowbirds and screaming

623 cowbirds while searching for host nests. Auk 131:610–618. https://doi.org/10.1642/AUK-14-54.1

624

625 Schielzeth H (2010) Simple means to improve the interpretability of regression coefficients. Methods Ecol Evol

626 1:103–113. https://doi.org/10.1111/j.2041-210X.2010.00012.x

627

628 Seel DC (1973) Egg-laying by the cuckoo. Brit Birds 66:528–535

629

630 Soler M, Palomino JJ, Martínez JG, Soler JJ (1995) Communal parental care by monogamous magpie hosts of

631 fledgling great spotted cuckoos. Condor 97:804–810

632

633 Soler M, Soler JJ, Martínez JG (1998) Duration of sympatry and coevolution between the great spotted cuckoo

634 (*Clamator glandarius*) and its primary host, the magpie (*Pica pica*). In: Rothstein SI, Robinson S (eds) Parasitic

635 birds and their hosts: studies on coevolution. Oxford University Press, New York, pp 113–142

636

637 Soler M, Soler JJ, Martínez JG, Møller AP (1994) Micro-evolutionary change in host response to a brood

638 parasite. Behav Ecol Sociobiol 35:295–301. https://doi.org/10.1007/BF00170710

639

640 Spottiswoode CN (2013) A brood parasite selects for its own egg traits. Biol Lett 9:20130573.

641 https://doi.org/10.1098/rsbl.2013.0573

642

643 Šulc M, Hughes AE, Troscianko J, Štětková G, Procházka P, Požgayová M, Piálek L, Piálková R, Brlík V, Honza M

644 (2021) Automatic identification of bird females using egg phenotype. Zool J Linn Soc-Lond 195:33–44.

645 https://doi.org/10.1093/zoolinnean/zlab051

646

647 Šulc M, Procházka P, Čapek M, Honza M (2016) Common cuckoo females are not choosy when removing an egg

648 during parasitism. Behav Ecol 27:1642–1649. https://doi.org/10.1093/beheco/arw085

649

650 Šulc M, Troscianko J, Štětková G, Hughes AE, Jelínek V, Čapek M, Honza M (2019) Mimicry cannot explain

651 rejection type in a host–brood parasite system. Anim Behav 155:111–118.

652 https://doi.org/10.1016/j.anbehav.2019.05.021

653

654 Trine CL (2000) Effects of multiple parasitism on cowbird and wood thrush nesting success. In: Smith JNM, Cook

655 TL, Rothstein SI, Robinson SK, Sealy SG (eds) Ecology and management of cowbirds. University of Texas Press,

656 Austin, pp 135–144

657

658 Trnka A, Prokop P (2012) The effectiveness of hawk mimicry in protecting cuckoos from aggressive hosts. Anim

659 Behav 83:263–268. https://doi.org/10.1016/j.anbehav.2011.10.036

660

661 Troscianko J, Stevens M (2015) Image calibration and analysis toolbox–a free software suite for objectively

662 measuring reflectance, colour and pattern. Methods Ecol Evol 6:1320–1331. https://doi.org/10.1111/2041-

663 210X.12439

664

665 Tuero DT, Fiorini VD, Reboreda JC (2007) Effects of shiny cowbird *Molothrus bonariensis* parasitism on different

666 components of house wren *Troglodytes aedon* reproductive success. Ibis 149:521–529.

667 https://doi.org/10.1111/j.1474-919X.2007.00676.x

668

669 Ursino CA, Strong MJ, Reboreda JC, Riehl C (2020) Genetic patterns of repeat and multiple parasitism by

670 screaming cowbirds, a specialist brood parasite. Anim Behav 167:177–183.

671 https://doi.org/10.1016/j.anbehav.2020.07.012

672

673 van den Berg CP, Troscianko J, Endler JA, Marshall NJ, Cheney KL (2020) Quantitative Colour Pattern Analysis

674 (QCPA): A comprehensive framework for the analysis of colour patterns in nature. Methods Ecol Evol 11:316–

675 332. https://doi.org/10.1111/2041-210X.13328

676

677 Wyllie I (1975) Study of cuckoos and reed warblers. Brit Birds 68:369–378

678

679 Wyllie I (1981) The cuckoo. Batsford, London

680

681 Yang C-C, Li D-L, Wang L-W, Liang G-X, Zhang Z-W, Liang W (2014) Geographic variation in parasitism rates of

682 two sympatric cuckoo hosts in China. Zool Res 35:67–71. https://doi.org/10.11813/j.issn.0254-5853.2014.1.067

683

684 Yang C, Stokke BG, Antonov A, Cai Y, Shi S, Moksnes A, Røskaft E, Møller AP, Liang W, Grim T (2013) Host

685 selection in parasitic birds: are open-cup nesting insectivorous passerines always suitable cuckoo hosts? J Avian

686 Biol 44:216–220. https://doi.org/10.1111/j.1600-048X.2013.00123.x

687

688   Zölei A, Bán M, Moskát C (2015) No change in common cuckoo *Cuculus canorus* parasitism and great reed

689   warblers' *Acrocephalus arundinaceus* egg rejection after seven decades. J Avian Biol 46:570–576.

690   https://doi.org/10.1111/jav.00673

691

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693 **Figure captions**

694

695 Fig. 1 Inter-annual variation in (A) the number of host nests (grey bars) and parasitism rate (black line) and (B)

696 in the number of cuckoo eggs found per one host nest. Data are based on daily nest checks prior to and during

697 host egg laying in 2007–2021 and on continual video-recording in 2016, 2018–2020

698

699 Fig. 2 Relationship between the annual rate of multiple parasitism and (A) annual parasitism rate and (B) the

700 total number of cuckoo eggs found each year. Data are based on daily nest checks prior to and during host egg

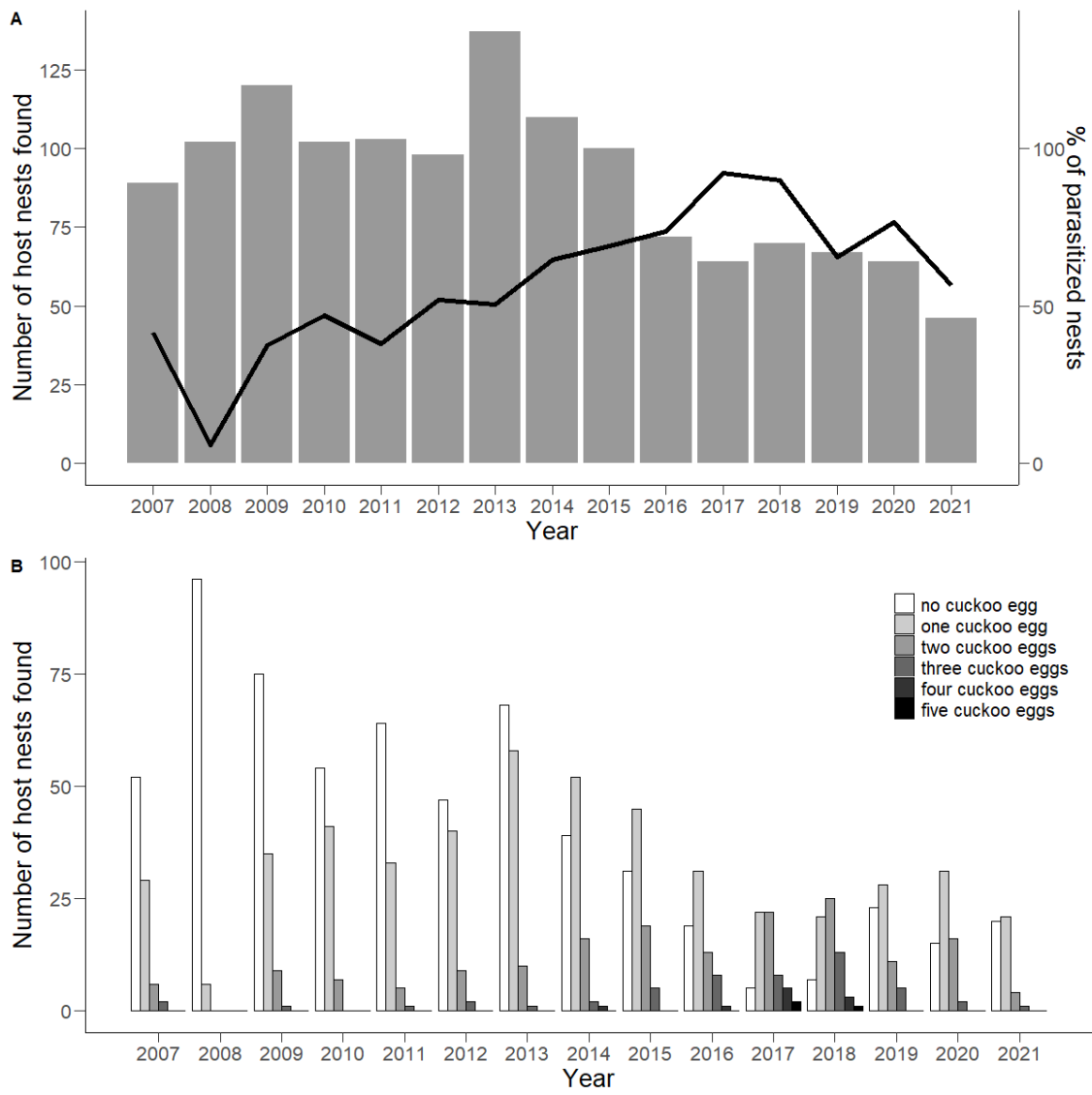701 laying in 2007–2021 and on continual video-recording in 2016, 2018–2020

702

703 Fig. 3 Number of cuckoo eggs (N = 220) found in host nests (N = 135) relative to host laying sequence (day 1 =

704 host clutch initiation). Negative days represent cuckoo eggs laid before host clutch initiation. Data are based on

705 continual video-recording in 2016, 2018–2020
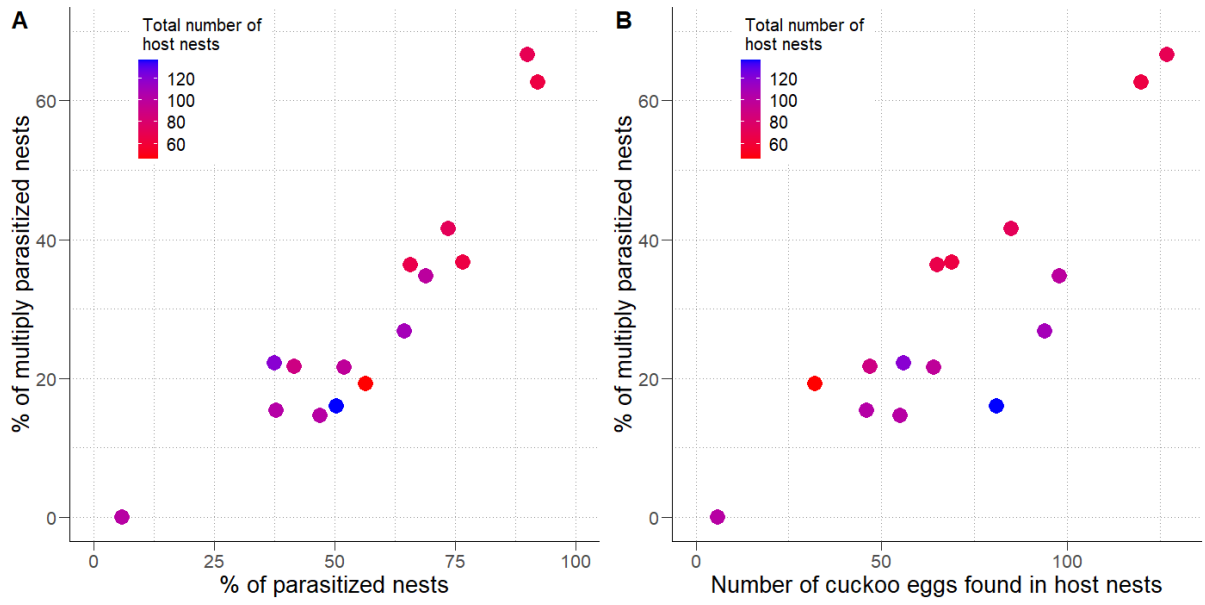
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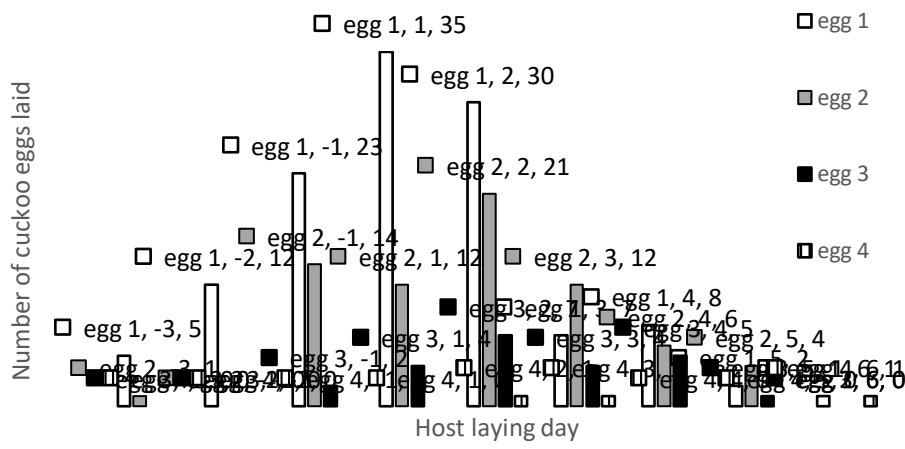
708   **Fig. 1**

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713   **Fig. 2**

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Fig. 3