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Molecular evidence for extra-pair paternity and intraspecific brood parasitism in the Black-headed Gull

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1 **MOLECULAR EVIDENCE FOR EXTRA-PAIR PATERNITY AND INTRASPECIFIC**
2 **BROOD PARASITISM IN THE BLACK-HEADED GULL**

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21 Running headline: EPP and ISBP in the Black-headed Gull

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30 **Abstract**

31 Social monogamy is common among birds, while genetic monogamy is supposed to be
 32 rare. I investigated the genetic mating system of the Black-headed Gull *Larus ridibundus*,
 33 where, as in seabirds and most other long-lived and socially monogamous birds, extra-
 34 pair paternity (EPP) is typically rather infrequent. Parentage was determined using six
 35 microsatellite markers for 79 chicks from 30 broods. In this study population, I found
 36 evidence of allelic inconsistencies between putative parents and chicks in 43% of nests,
 37 resulting from both EPP and intraspecific brood parasitism (ISBP). Extra-pair paternity
 38 was detected in 33 % (10/30) of broods, and 20% (16/79) of all nestlings were sired by
 39 extra-pair males. Furthermore, 9% (7/79) of chicks out of 5 nests (17%) were not the
 40 offspring of either member of the pair, indicating ISBP. These findings reveal a moderate
 41 rate of ISBP and a high rate of EPP compared with other related species, and shows that
 42 Black-headed Gulls successfully participate in extra-pair copulations.

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44 **Key words** Black-headed Gull, genetic mating system, extra-pair paternity, intraspecific
 45 brood parasitism, *Larus ridibundus*

46

47 **Zusammenfassung**

48 **Molekularer Nachweis von Fremdvaterschaft und intraspezifischem Brutparasitismus**
 49 **bei der Lachmöwe *Larus ridibundus***

50 Soziale Monogamie ist bei Vögeln weitverbreitet, während genetische Monogamie vermutlich
 51 selten ist. Ich habe das genetische Paarungssystem der Lachmöwe *Larus ridibundus*
 52 untersucht, bei der, wie bei Seevögeln und den meisten anderen langlebigen und sozial
 53 monogamen Vögeln, Fremdvaterschaft (EPP) normalerweise eher selten ist. Die Elternschaft
 54 wurde mittels sechs Mikrosatellitenmarkern für 79 Küken aus 30 Brutten ermittelt. In der
 55 untersuchten Population fand ich Belege für Allelinkonsistenzen zwischen Putativeltern und
 56 Küken in 43 % der Nester, was sowohl auf EPP als auch auf intraspezifischen
 57 Brutparasitismus (ISBP) zurückzuführen war. Fremdvaterschaft wurde in 33 % (10/30) der
 58 Brutten nachgewiesen, und 20 % (16/79) aller Nestlinge wurden von Fremdvätern gezeugt.
 59 Außerdem stammten 9 % (7/79) der Küken aus fünf Nestern (17 %) weder von der Mutter
 60 noch vom Vater ab, was auf ISBP hindeutet. Diese Ergebnisse lassen eine im Vergleich zu

61 anderen verwandten Arten mittlere ISBP-Rate und eine hohe EPP-Rate erkennen und zeigen,
62 dass Lachmöwen erfolgreich Kopulationen außerhalb des Paarbundes eingehen.

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76 **Introduction**

77 The application of molecular techniques to avian mating systems has revealed
78 striking variation in the incidence of alternative reproductive strategies such as extra-pair
79 paternity (EPP) and intraspecific brood parasitism (ISBP) (Birkhead and Møller 1992;
80 Petrie and Kempenaers 1998; Arnold and Owens 2002). Even though many hypotheses
81 have been proposed to explain this variation (Petrie and Kempenaers 1998; Møller and
82 Ninni 1998; Arnold and Owens 2002; Griffith et al. 2002; Westneat and Stewart 2003;
83 Neudorf 2004), there is still inconsistency in the results. The strongest correlation with
84 EPP was found phylogenetic. High EPP rates are associated with high rates of adult
85 mortality and reduced parental care, while high ISBP rates are associated with high
86 fecundity rates (Arnold and Owens 2002; Bennett and Owens 2002). Variation at the
87 population or individual level is more likely to be based on differences in current
88 ecological and genetic factors (Petrie and Kempenaers 1998). Of ecological factors,
89 breeding density may be an important factor at the species level (Westneat and Sherman

90 1997). Colonially nesting species are predicted to have high EPP rates because proximity
91 to potential partners is high (Møller and Birkhead 1993).

92 The Black-headed Gull, like other gulls, is a long lived, colonially breeding species
93 with high adult survivorship (Cramp 1983). The standard clutch size of this species is
94 three eggs. Both females and males participate in parental care throughout incubation
95 and chick-rearing, which leads to social monogamy in this species (Cramp 1983). Given
96 these traits, a low frequency of EPP and ISBP could be expected.

97 The major goal of this study was to describe the rates of extra-pair parentage and
98 intraspecific parasitism in the Black-headed Gull.

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105 ***Materials and methods***

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107 ***Field methods and sampling***

108 The study was carried out in a colony of about 3 000 pairs of Black-headed Gulls at the
109 Jarohněvický pond in the south-eastern part of the Czech Republic (48°55'N 17°05'E).

110 Thirty families (chicks and both parents) were sampled during the breeding season 2004.

111 Adults were trapped with "walk-in-nest-traps" at randomly chosen nests from different
112 parts of the colony, including both the centre and edges, and ringed with metal rings for
113 identification. A sample of blood (approximately 50 µl) was taken from the tarsal vein of
114 each trapped individual and mixed with 150 µl of storage buffer (0.5M EDTA), for later
115 sex determination and paternity analysis.

116 The distribution of clutch sizes in the thirty sampled nests size was: 4 eggs (n=1), 3 eggs
117 (n=20), 2 eggs (n=7) and one egg (n=2). Eggs were individually marked with waterproof
118 ink on the shells, according to laying sequence. Each of the studied nests was enclosed

119 within a 1.6 cm mesh wire circle about 1 m in diameter and 40 cm high. This fence
120 prevented the escape of young chicks but allowed adults to access the nests.

121 On the first day after hatching, approximately 25 µl of blood was obtained from
122 the brachial or tarsal vein of chicks and mixed with 150 µl of storage buffer (0.5M EDTA).
123 Samples of embryos were collected from unhatched eggs. All samples were kept at –
124 20°C until DNA extraction.

125

126 ***Molecular determination of sex***

127 The sex of social parents was determined by a polymerase chain reaction that
128 amplifies an intron of the CHD1 genes on the sex chromosomes (Griffiths et al. 1998).
129 Genomic DNA was isolated from 10 µl of blood sample using the DNA Lego Kit (Top Bio)
130 following the manufacturer's protocol. The avian sexing primers 2550 F and 2718 R
131 (Fridolfsson and Ellegren 1999) were used in 10 µl PCR reactions. The PCR conditions
132 were as follows: 1µl of 10X PCR buffer (TaKaRa), 2 µM of each dNTP, 2.5 pmol of each
133 primer and 0.5 U of *Taq* polymerase (TaKaRa). About 150 ng of genomic DNA was used
134 as template. PCR was performed according to Griffiths et al. (1998) in a T3 Biometra
135 thermal cycler. The products were separated by electrophoresis for 45-60 min at 7-10
136 V/cm using a 3% agarose gel stained with ethidium bromide.

137

138 ***Parentage analyses***

139 Microsatellite typing was used to reveal the genetic relationships between parents and
140 young. Parentage was analysed using 6 polymorphic dinucleotide microsatellite loci (RBG
141 13, RBG 18, RBG 20, RBG 27, RBG 28, RBG 29) originally developed for the Red-billed
142 Gull *Larus novaehollandiae scopulinus* (Given et al. 2002). These six loci exhibited little
143 or no evidence of null alleles and showed sufficient levels of polymorphism (mean
144 heterozygosity = 0.77, mean number of alleles = 11.3), making them suitable for
145 paternity assessment in this species (combined exclusion probability: first parent = 0.98,
146 second parent = 0.998) (Table.1).

147 Genomic DNA was extracted from 10 µl of blood sample or from 25-35 mg of

148 embryo tissue (depending on type of tissue) from unhatched eggs. The 10 μ l PCR
149 reactions consisted of about 100 ng of template DNA, 0.4 units of *Taq* Polymerase
150 (TaKaRa), 1 μ l of 10x PCR buffer (TaKaRa), 2.5 μ M dNTPs and 2.5 pmol of each primer.
151 The forward primer was fluorescently labelled. The PCR conditions followed the protocol
152 described by Given et al. (2002).

153 Amplification products were run on an ABI Prism 3100 automatic sequencer, and
154 Gene Mapper software (Applied Biosystems Inc) was used to size the alleles. The number
155 of alleles, observed and expected heterozygosity, parentage exclusion probability for the
156 first and second parents, and theoretical frequency of null alleles for each locus were
157 estimated with CERVUS 3.0.3 software (Kalinowski et al. 2007) (Table. 1). Allele
158 frequencies were used to calculate the cumulative probability of resemblance P_{RCum} for
159 specific parent-offspring cases where ambiguous mismatches occurred (Ibarguchi et al.
160 2004).

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163 **Results**

164

165 I assessed paternity for 79 chicks and 60 adults from 30 broods. Offspring that
166 possessed a microsatellite allele that did not match the putative father at two or more
167 loci were considered extra-pair young (EPY), and offspring that did not share an allele
168 with either of their social parents were considered to be a case of intraspecific brood
169 parasitism. In 17 out of 30 sampled nests (57%), I found no case of allelic mismatch
170 between the offspring and their putative parents. Thus, all social parents were true
171 genetic parents in these nests. However, in 13/30 (43%) nests, allelic inconsistencies
172 between putative parents and chicks were detected, resulting from both EPP and ISBP.
173 More specifically, in 10/30 (33 %) nests I found that 17/79 (21.5%; 95% c.i.: 0.13 -
174 0.32) chicks shared the same allele at each locus with their mother, but were
175 mismatched with the social father in at least two loci (Table.2). The number of
176 mismatching loci in extra-pair chicks ranged from two to five loci (Table.3). In three

177 cases, where the chick and its putative father differed only in two loci, I estimated the
178 likelihood that mismatched loci were caused by mutation. Cumulative probabilities of
179 resemblance (P_{Rcum} ; Ibaraguchi et al. 2004) were calculated for these three chicks,
180 excluding loci with the mismatched alleles. In two cases, the probabilities of sharing
181 alleles by chance were so high (0.2×10^{-3} and 1.3×10^{-3}) that mismatches due to mutation
182 could be ruled out. In the third case, the cumulative probability of resemblance was
183 much lower (5×10^{-6}), suggesting that this was a case of mutation and the chick could be
184 reassigned as legitimate. I considered the remaining 16/79 (20%; 95% c.i.: 0.12-0.31)
185 chicks to be extra-pair, since in all cases social mothers were also true genetic mothers.

186 Moreover, in two of the ten nests with EPY and in three other nests I found chicks
187 which did not match either of their social parents. Altogether 7/79 (9%; 95% c.i.: 0.04-
188 0.17) chicks out of 5/30 (17%) nests were considered to result from intraspecific brood
189 parasitism (Table.2). The frequency of mismatching loci in these parasitic chicks ranged
190 from 3 to 6 loci (Table.3).

191 Finally, in another five chicks I detected differences between chicks and parents at
192 a single locus; in four cases chicks differed from their social father and in one case the
193 chick mismatched at one locus with their mother. The cumulative probabilities of
194 resemblance were calculated for these five chicks, excluding loci with the mismatched
195 alleles. In four of five cases, probabilities of sharing alleles by chance were so low ($4.08 \times$
196 10^{-6} to 7.01×10^{-7}) that mutation is the most probable reason for the mismatches. In the
197 last case, the probability of sharing alleles was higher (1×10^{-2}) due to the male and
198 chick sharing common alleles (Table.3). All those five chicks were considered as within
199 pair offspring.

200

201

202 **Discussion**

203

204 Black-headed Gulls in the study population successfully participate in extra-pair
205 copulations – 33% of nests contained at least one extra-pair young (EPY). This level of

206 EPP is relatively high in comparison to related species and other colonially breeding
207 seabirds (e.g. Mauck et al. 1995, Gilbert et al. 1998, Lorentsen et al. 2000, Griggio et al.
208 2004). Previously measured variation in EPF rates for Laridae species has been low,
209 ranging from zero in the Western Gull (*Larus occidentalis*; Gilbert et al. 1998), Black-
210 legged Kittiwake (*Rissa tridactyla*; Helfenstein et al. 2004), Common Tern (*Sterna*
211 *hirundo*; Griggio et al. 2004) and Little Auk (*Alle alle*; Lifjeld et al. 2005), to 8.3 % in
212 Common Gull (*Larus canus*; Bukacinska et al. 1998). These findings are in line with the
213 prediction of low EPP rates in species with a long reproductive lifespan, such as seabirds.

214 In contrast, my data suggest that Black-headed Gulls have an unexpectedly high
215 rate of EPP. Extra-pair paternity could arise by two means, either as the result of extra-
216 pair copulations (EPC), or rapid mate-switching (Birkhead and Møller, 1992, Martins et al.
217 2002). In gulls, EPCs are common (Mills 1994, Bukacinska et al. 1998). In Black-headed
218 Gulls, copulation activity has not yet been studied in detail; Frantová (2007) found a low
219 frequency of forced copulations (1.8%), but did not distinguish between within-pair and
220 unforced extra-pair copulations.

221 I collected no behavioural data on mate-switching, so that I can not exclude the
222 possibility that the two nests where all three chicks were found to be sired by other than
223 the social father (Table.2) could be cases of mate-switching or, alternatively, cases of
224 putative father infertility. The replacement of a mate might happen rapidly due to a large
225 supply of potential mates and strong competition for mates during the early stage of
226 breeding. In the remaining 8 nests, one (5 nests) or two (3 nests) EPYs were detected,
227 probably resulting from successful EPCs (Table.2). However, to unequivocally explain this
228 high rate of EPP, other, especially behavioural studies will be necessary. Furthermore,
229 the accuracy of the estimate could be affected by used sample size (Griffith et al. 2002).
230 Griffith et al. (2002) suggest that approximately 200 offspring is a reasonable sample
231 size limiting the magnitude of error around the estimate. The magnitude of error around
232 my estimate of EPP level in studied population is 19%, which suggest that further
233 sampling would increased the accuracy of the estimate.

234 Moreover, I found a quite high rate of ISBP, especially considering the life-history

235 strategy of gulls. In five (17%) out of 30 sampled nests, at least one chick was found
236 that did not match either of their social parents. This rate of ISBP should not be affected
237 by possible chick adoption, since the studied nests were enclosed with mesh wire and
238 chicks were sampled within a couple of hours after hatching. Multiple parentage in these
239 clutches most probably occurred through nest parasitism. The possibility that some of the
240 mixed clutches represented female-female pairs or quasi-parasitism can be excluded,
241 since the social parents trapped at each nest were always a male and female, and neither
242 of them matched potentially parasitic chicks.

243 Among 61 species of gulls, intraspecific parasitism has been documented in
244 another four species (*Larus argentatus*, *L. canus*, *L. delawarensis* and *L. minutus*; in
245 Yom-Tov 2001 and Duda et al. 2008) as well as in the Black-headed Gull (Duda et al.
246 2008, Ležalová-Piálková and Honza 2008). Duda et al. (2008) revealed the maternity in
247 a population of Black-headed Gulls in Poland by protein fingerprinting, and found multiple
248 maternity in 34% of nests with >1 egg. These results are much higher than those
249 obtained from a previous study of ISBP based on the regular monitoring of nests in the
250 same Black-headed Gull population as the current study, (Ležalová-Piálková and Honza
251 2008). In this previous analysis, we found a parasitism rate of 10%, which now seems to
252 be an underestimation. These studies allow an interesting comparison between three
253 different methodological approaches to the question of ISBP, showing that a molecular
254 approach is essential for finding the real frequencies of ISBP in birds (Grønstøl et al.
255 2006).

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338

339 **Table 1**

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341 **Table.1** Polymorphism data for six microsatellite markers in the Black-headed Gull.
 342 Values are based on the genotypes of 60 parents and calculated using CERVUS 3.0.3. k -
 343 the number of alleles, size (bp)- range of allele size in base-pairs, He - expected
 344 heterozygosity, Ho - observed heterozygosity, F_{null} - estimated frequency of the null
 345 allele, $NE-1P$ - non-exclusion probability for the first parent, $NE-2P$ - non-exclusion
 346 probability for the second parent.

Locus	k	size (bp)	He	Ho	F_{null}	$NE-1P$	$NE-2P$
RBG 13	9	205-223	0.82	0.86	0.02	0.54	0.36

RBG 18	6	170-180	0.39	0.35	0.09	0.93	0.8
RBG20	16	169-203	0.92	0.9	0.01	0.29	0.17
RBG 27	21	185-235	0.91	0.89	0	0.33	0.2
RBG 28	7	159-173	0.76	0.75	0	0.64	0.46
RBG 29	9	125-141	0.81	0.9	0.06	0.56	0.38

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356 **Table 2**357 **Table.2** Distribution of chicks with extra-pair paternity (EPP) and parasitic chicks (ISBP)

358 across the nests. n = number of analysed nests.

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		No. of chicks with extra- pair parentage in nest			Total (chick/nests)	n
		1	2	3		
No. of nests with determined	EPP	6	2	2	16/10	30
	ISBP	4	-	1	7/5	30

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365 **Table 3**

366 **Table.3** Frequency of chicks with different number of mismatched loci. PR_{cum} -
 367 cumulative probability of resemblance of sharing alleles by chance for parent-offspring
 368 cases with low number of mismatching loci, nd – not defined.

No. of mismatched loci	No. of chicks	PR_{cum}	Classified as
1	5	1×10^{-2} to 7.01×10^{-7}	true parentage
2	1	5×10^{-6}	true parentage
2	2	0.2×10^{-3} and 1.3×10^{-3}	EPP
> 3 (max. 5)	14	nd	EPP
> 3 (max. 6)	7	nd	ISBP

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