The effects of parental age difference on the offspring sex and fitness of European blackbirds

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Background. Many studies of birds have indicated that offspring sex ratios can vary with environmental and parental traits. On the basis of long-term research, we first evaluated the possible influence of parental age difference and brood characteristics on offspring sex and fitness in multi-brooded Blackbirds *Turdus merula*. **Methodology.** The study was conducted in the city-centre Stefan Żeromski Park in Szczecin, NW Poland, where the local population of Blackbirds has been studied since 1996. Data on the offspring sex and fitness were collected in five years, 2005-2007 and 2016-2017. During the breeding season we inspected the study area to locate the pairs' territories and to track their nests and clutches. **Results.** We found that the overall sex ratio did not differ statistically from 50:50, but that younger females bonded with older mates did tend to produce more sons, probably because of the greater fitness of male descendants. Accordingly, the sons' breeding success increased with the father's age, but this relationship was close to non-linear, which may indicate that the transgenerational effect of paternal senescence could negatively affect progeny fitness despite the high-quality of older fathers. Older females mated with younger males produced more daughters, which could have been due to the lesser attractiveness of the males and the mothers' poorer condition caused by accelerated senescence. We found that neither offspring hatching sequence nor hatching date or clutch sequence were significant for sex determination. **Conclusions.** We consider that in our Blackbird population, parental age could make a more significant contribution to shaping offspring sex and reproductive success.

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2 **blackbirds**

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21 **Abstract**
22
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30 breeding season we inspected the study area to locate the pairs' territories and to track their nests
31 and clutches.
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34 the greater fitness of male descendants. Accordingly, the sons' breeding success increased with
35 the father's age, but this relationship was close to non-linear, which may indicate that the
36 transgenerational effect of paternal senescence could negatively affect progeny fitness despite
37 the high-quality of older fathers. Older females mated with younger males produced more
38 daughters, which could have been due to the lesser attractiveness of the males and the mothers'
39 poorer condition caused by accelerated senescence. We found that neither offspring hatching
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42 significant contribution to shaping offspring sex and reproductive success.
43

44 **Introduction**

45 Fisher's theory states that natural selection should promote investment in equal sex ratios of
46 offspring: any deviation from equality should not be selected because of negative frequency-
47 dependent selection (simple deviation from equality cancels out in a population, so the best
48 strategy is to produce offspring with an equal sex ratio) (Fisher, 1930). But according to another
49 model, the Trivers-Willard hypothesis (Trivers & Willard, 1973; Ewen, Cassey & Møller, 2004;
50 Cassey, Ewen & Møller, 2006; Booksmythe et al., 2017), selection under different maternal
51 conditions should promote deviations from the equal offspring sex ratio. This is because the
52 maternal condition more profoundly affects sons which, raised in good conditions, will have a
53 superior breeding success than females raised in the same conditions (Trivers & Willard, 1973).
54 Hence, a mother in better condition should invest more in sons; but if the mother is in poor
55 condition, daughters ought to be preferred (Myers, 1978; Cockburn, Legge & Double, 2002;
56 Merkling et al., 2015). This sex bias could also be enhanced by male sexually-selected traits: the
57 attractive ornaments of males are signals of their high quality, so females should produce sons
58 with these high-quality fathers but daughters from low-quality fathers (Burley, 1981). There is
59 evidence that the offspring sex ratio in birds can vary adaptively according to maternal condition,
60 mate quality, mate competition, resource availability, seasonal effects, hatching order, clutch
61 size, parental compatibility and parental age (Ewen et al. 2004, Booksmythe et al. 2017, and
62 references therein). However, meta-analyses find weak support, if any, for the Trivers-Willard
63 hypothesis (Ewen, Cassey & Møller, 2004; Cassey, Ewen & Møller, 2006; Booksmythe et al.,
64 2017).
65   Female birds are often used as a model for testing primary sex ratio manipulation. They
66 can manipulate the sex of their offspring before fertilisation: females can produce Z ("male") or

67 W ("female") gametes, but males produce sperm with only Z gametes. Therefore, sex is
68 determined during meiosis in the female gonads (Navara, 2018). There is also another
69 explanation: chimeric embryos with competing cells contain those sex chromosomes (Tagirov &
70 Rutkowska, 2013). Moreover, birds can alter the secondary sex ratio (after egg laying) via
71 embryo mortality by manipulating the incubation temperature (DuRant et al., 2016) or
72 differential allocation of parental care to chicks of different sexes, i.e. females can exert sex-
73 differential investment in eggs, males and females can use sex-differential parental investment
74 rules when feeding chicks (Lessells, 2002). There are many factors that might reflect or influence
75 sex-specific parental care that can have effects on offspring fitness, i.e. factors related to the
76 parents' quality (condition, age, breeding experience, body size, attractiveness), external and
77 social environment quality (timing of breeding, weather, food availability) or the number and
78 asymmetry among the chicks (clutch size, hatching asynchrony; e.g. reviews in Hasselquist &
79 Kempenaers, 2002; Komdeur & Pen, 2002).
80   In this study, we examined possible offspring sex bias in an open-cup nesting passerine –
81 the European Blackbird *Turdus merula* (henceforth Blackbird). There were several reasons for
82 this: (1) Blackbirds are socially monogamous, dimorphic birds (the only *Turdus* species with
83 clear differences in plumage colouration), where adult males are bigger than adult females
84 (Piliczewski, Jankowiak & Wysocki, 2018); female mating preference could therefore depend on
85 a male phenotypic trait, or else a single male trait, such as age, could affect male mating
86 opportunities; (2) high-quality males of this species engage in extra-pair copulations (Wysocki &
87 Halupka, 2004); (3) the population studied exhibits many different breeding strategies in order to
88 maximise breeding success (Wysocki, 2004, 2005, 2006; Wysocki & Walasz, 2004; Wysocki &
89 Jankowiak, 2018); (4) egg size in this species has been found to be sexually dimorphic – larger
90 eggs contain male embryos (Martyka et al., 2010) – and hatching in this species is highly
91 asynchronous (Magrath, 1989), so we can expect a relationship between offspring sex and
92 hatching sequence, and older females should produce smaller eggs because of their poor
93 condition due to senescence; (5) chick productivity in the target urban population is low and
94 lifetime breeding success is subject to considerable variance: during a 21-year study, just 7% of
95 males and 16% of females raised 50% of all fledglings; the less numerous, oldest individuals are
96 the birds with the highest lifetime breeding success (Wysocki et al., 2019, Zyskowski, 2015). A
97 few studies have shown that age is a significant factor influencing reproductive life-history traits
98 of blackbirds (Desrochers, 1992a,b; Desrochers & Magrath, 1993; Streif and Rasa, 2001). After
99 the peak of, senescence comes into play. Senescence is defined as a decrease in the body's
100 physiological performance, resulting in a decline in survival rate or breeding success with age
101 (Monaghan et al., 2008). Our earlier studies indicated that a blackbird's reproductive
102 performance reach the peak at 5–6 years old (i.e. birds ability to breed earlier) then declines (the
103 oldest birds lived to 12-13 years) as the process of senescence sets in (Jankowiak & Wysocki,
104 2015).
105   Several studies have shown that birds can manipulate the offspring sex ratio by
106 controlling hatching asynchrony (Badyaev et al., 2002; Tryjanowski et al., 2011). Our principal
107 aim was to test this hypothesis. Accordingly, we predicted that first-hatched eggs should more
108 often be males, assuming that the first chick would be in better condition because it experiences
109 less competition since it hatches first and it was fed earlier. We also wanted to test whether other
110 brood characteristics, such as the hatching date and clutch order in the breeding season, could
111 influence the sex ratio. Thus, we predicted that earlier-hatched offspring and/or first clutches in
112 the season should be related to the higher probability of sons. This should be so because the

113 earlier hatching time in the breeding season allows the chick more time to grow up in good
114 conditions and to acquire the foraging skills necessary to survive winter (Zyskowski, 2015). This
115 is the so-called "silver spoon" effect (Van De Pol et al., 2006), where the impacts of natal
116 condition can be profound later in life. In late broods, mothers should invest in daughters
117 because of their poorer condition later in the season and possibly lower survival rates (Verhulst
118 & Nilsson, 2008; Rodríguez et al., 2016). Hence, it is better to invest in the "costly" sex early
119 and the "cheaper" sex later in the breeding season.
120   The second main aim was to test the possible impact of parental age on offspring sex. We
121 used parental age as a proxy of individual quality. Older males are better survivors, have greater
122 breeding experience and can provide greater parental care: all this may reflect the genetic quality
123 of males with indirect effects on offspring fitness, e.g. the sons of "good gene" fathers
124 (Weatherhead & Robertson, 1979; Hansen & Price, 1995; Kokko, 1998) are more attractive
125 because they inherit genes that make them better parents and survivors. If an older individual
126 male partner possessed "good genes", the female should manipulate the brood ratio towards
127 sons; conversely, if the mother were much older than the father, more daughters should be
128 expected in the brood. Moreover, older females should produce more daughters owing to their
129 poorer condition caused by senescence. Longitudinal studies of senescence accumulate rapidly
130 from wild animal populations. However, it is largely unknown how senescence in birds
131 contributes to offspring sex ratio and declines in reproductive value (Nussey et al., 2008;
132 Bouwhuis et al. 2012). In view of this, we hypothesised that the age differences between the
133 parents should affect the offspring sex. To evaluate the transgenerational effect and confirm that
134 older males are genetically superior, we assessed whether paternal age affected the breeding
135 success of their offspring.
136

Methods

138 The data were collected in the city-centre Stefan Żeromski Park in Szczecin, NW Poland
139 (53°260'N, 14°330'E). The canopy is dominated by oaks *Quercus* sp., limes *Tilia* sp., Beech
140 *Fagus sylvatica* and Horse Chestnut *Aesculus hippocastanum*. The shrub layer consists mainly of
141 Common Yew *Taxus baccata*, but it is not very dense and covers only about 7% of the park's
142 total area (21 hectares).
143   Due to the fact that research on blackbirds has been going on there since 1997, over 90%
144 of the birds in this population were ringed. Birds were ringed as chicks in their nests (between
145 the 6th and 10th day of life), or just after fledging, while they were still in the care of their parents,
146 as independent individuals trapped in mist-nets. The birds were sexed and aged (Svensson,
147 1992). The retrapping or resighting of ringed birds in subsequent years enabled them to be aged
148 precisely. We consistently use the calendar year of a bird's life. The Blackbird is a multi-brooded
149 species: a female can make from three to six breeding attempts from the beginning of March to
150 the end of August, three (exceptionally four) of which may be successful (Wysocki, Cholewa &
151 Jankowiak, 2018). Females lay 1-6 eggs, mostly 3-5 (mean: 4.2 ± 0.9 s.d., n = 459). The mean
152 yearly success per pair – all broods in the year of a particular pair (n = 1284, measured as the
153 total number of fledglings to have left the nests during the breeding season) – was 1.5 ± 2.2 s.d.
154 fledglings.
155   In this paper, we used data collected during 2005-2007 and 2016-2017 seasons. During the
156 breeding season (March-August), 1-3 persons inspected the study area every day to locate the
157 pairs' territories and to track their nests and clutches. This enabled us to obtain exact hatching

158 date and numbers of fledglings which left the nests (our fitness measure) in the breeding season.
159 The exact date of hatching was determined by observing hatchlings in the nest or the male who
160 first brought food for the chicks and gave it to the female first, then the female would feed the
161 chicks (in nests located high). Observations of the nests in which the chicks were to hatch were
162 carried out several days before the planned hatching so as not to miss it. The male that fed the
163 chicks was referred to as the female's social partner and the father of the chicks (we realized that
164 the nest could contain half-siblings, but unfortunately we do not have data on paternity in this
165 population). We used relative chick mass on the day of ringing as a measure of hatching order.
166 This method of determining the hatching sequence is sufficient in the case of our blackbird's
167 population. In support of ours statement in 90% of broods the weight on the day of ringing
168 reflects the hatching sequence (Wysocki, Cholewa, unpublished data).
169   Growth feathers were taken from nestlings while they were being ringed (between the 6th
170 and 10th day after hatching). The feathers were taken with tweezers and each was placed
171 individually in a plastic bag. Plastic bags were marked with the mother's, father's and chick's ID
172 and then placed in the fridge. This involved a total of 399 chicks from 132 nests in the breeding
173 seasons 2005 (1 brood), 2006 (24 broods), 2007 (22 broods), 2016 (49 broods) and 2017 (36
174 broods). Part of these data (from years 2006 and 2007) were analysed and published in Nowacki
175 et al. (2016) but due to the insufficient amount of data, only the simplest statistical analyzes were
176 performed in this article, which showed no differences in the sex ratio. Therefore, we decided to
177 add these data set to the new analysis and improve our preliminary results.
178   We confirm that all the methods were carried out in compliance with the relevant
179 guidelines and regulations. All experimental protocols were approved by an institutional and
180 licensing committee as detailed hereafter. Bird capture and ringing took place under the
181 supervision of Dariusz Wysocki (ringing license No. 390/2018 from the Polish Academy of
182 Sciences). Marking (combinations of four colour rings) was also carried out by permission of the
183 Polish Academy of Sciences. Permits for the survey as well as for feather and blood sampling
184 were obtained from the Local Ethics Committee in Szczecin (No. 6/06 dated 26.02.2006) and the
185 Local Ethics Committee in Poznań (No. 6/2014 dated 23.04.2014; Poland) (legal basis:
186 Ordinance of the Minister of Science and Higher Education of 5 May 2015).
187
188 *DNA isolation*
189 DNA was extracted from the feathers of Blackbird chicks using a High Pure PCR Template
190 Preparation Kit (Roche Diagnostics). The concentrations and quality of the isolates were
191 determined by agarose gel electrophoresis and spectrophotometry (NanoDrop 2000C; Thermo
192 Scientific).
193
194
195 *PCR reaction*
196 PCR reactions were carried out in the presence of primers P2
197 (5'TCTGCATCGCTAAATCCTTT3') and P8 (5'CTCCCAAGGATGAGRAAYTG3') (Griffiths
198 et al., 1998). The PCR reaction mix (15 µl) consisted of 1x PCR reaction buffer with Mg^{2+}, 2.0
199 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer, 0.75U RedAllegro *Taq* polymerase
200 (Novazym, Poland) and 50 ng DNA. The CHD1 gene amplification reactions were performed in
201 a T100™ Thermal Cycler (Bio-Rad) in 34 cycles, according to the following thermal profile:
202 initial denaturation at 94°C for 5 minutes, then at 94°C – 1 minute, 54°C – 1 minute, 72°C – 1
203 minute, and final elongation at 72°C for 5 minutes (Dybus et al., 2009).

204   The PCR products were separated on 2.5% agarose gel with ethidium bromide added in TAE
205   buffer. Gel Doc^{TM} XR+ system was used (Bio-Rad) to visualise and document the results.
206

207   *Sex identification*
208   The sex of Blackbird nestlings was identified by the presence of amplification products in
209   specific areas of the genes CHD1-Z and CHD1-W, located respectively on the Z and W sex
210   chromosomes. The amplicons obtained were sized using Quantity One 1-D Analysis Software
211   Version 4.6.5 (Bio-Rad) in relation to the molecular weight standard Nova 100 bp DNA Ladder
212   (Novazym, Poland).
213   In the case of homozygous males (genotype ZZ), single bands were identified on the
214   electrophoretic image, about 356 bp in size, whereas for heterozygous females (genotype ZW) an
215   additional CHD1-W gene amplification product (391 bp) was observed. The difference in the
216   size of the amplified fragments of genes CHD1-W and CHD1-Z was approximately 35 bp.
217

218   *Statistical analyses*

219   In this data set, we analysed the occurrence of a chick's sex in the nest that we alternatively
220   called the probability of being male/female. Offspring sex was the dependent variable, and
221   parental age difference and brood characteristics were the explanatory variables. A parental age
222   difference of zero implies the same age of the father and mother; a negative value indicates that
223   the mother was older than the father, while a positive one shows that the father was older than
224   the mother. The brood characteristics were as follows: clutch sequence, i.e. the clutch order in a
225   given breeding season; offspring hatching sequence, i.e. the order in which the offspring hatched
226   in the nest; hatching date, i.e. the day when a nestling hatched. For this analysis, we used 382
227   sexed chicks (a smaller number than the total number of sexed birds because of missing
228   information relating to parental age). We also performed analyses with year as an additional
229   random factor and further analyses where, instead of the age difference, we took the ages of the
230   father and mother and their interaction into consideration (the variation inflation factor indicates
231   no multicollinearity; VIF = 1.11). In this last case, we analysed brood characteristics rather than
232   parental age as the explanatory variable. To check whether there were differences among pairs
233   where males and females were the same age (109 chicks), we analysed offspring sex depending
234   on parental age and brood characteristics (clutch sequence, offspring hatching sequence and
235   hatching date). We applied a generalised linear mixed model with binomial error distribution
236   because we had sexed each chick in the nest, so for the analysis of a single chick, the effect of
237   nest and hatching sequence should be controlled as a random slope effect (Offspring hatch
238   sequence/ Nest id). We also evaluated the influence of the parental age difference, clutch
239   sequence and hatching date on the brood sex ratio (measured as the number of males/total
240   number of offspring). But as the results were of a similar quality, we merely described the model
241   with offspring sex as the dependent variable.
242   To confirm that older fathers were high-quality individuals, we evaluated the relationship
243   between the father's age and his sons' breeding success (calculated as the total number of
244   fledglings produced in a given breeding season). As a control, we related the mother's age to her
245   sons' reproductive success. In another control, we performed a second, analogous model in
246   which the daughters' breeding success was chosen instead of the sons' breeding success. In both
247   analyses we used a generalised linear mixed model with negative binomial error distribution. As

248 sons and daughters frequently bred more than once, we controlled for this by adding a simple
249 random intercept of individual (Id).
250   The analyses were performed using R statistical software (R Development Core Team,
251 2014); the mixed models were performed in the lme4 package (Bates et al., 2014).
252

Results

254 During a total of four years of research, 399 Blackbird chicks from 132 broods (mean brood size
255 = 3.02 ± 0.10 s.e.) were sexed. The male-to-female ratio was 204 (51.1%): 195 (48.9%), a result
256 not statistically different from 50:50 ratios (Goodness of Fit Test: $\chi^2 = 0.206$, df = 1, p = 0.650).
257   We found that neither offspring hatching sequence (Table 1 and 2) nor hatching date or
258 clutch sequence were significant as regards sex determination (Table 2). Only the difference in
259 the parents' age affected this aspect (Table 2, Fig. 1). When the father was older than the mother,
260 the probability of producing male offspring was much higher than when the parents were of the
261 same age. In contrast, when the female was older than the male, the probability of having a
262 daughter was higher. In the model with year as the random factor, the results were similar (only
263 the age difference was significant: Chi2 = 6,706; p < 0,01; for the remaining variables p > 0.3),
264 so we decided to show only the results of previous analyses. There was no difference in offspring
265 sex occurrence between pairs where males and females were the same age (for all variables p >
266 0.2). In the model to which we added the mother's and father's ages as explanatory variables, we
267 found that both age and interaction of parental age were nonsignificant (for all variables p > 0.3).
268 After removing the interaction from the model, the mother's age was negatively related to the
269 probability of having sons' in the brood ($\beta_{mother's\ age} = -0.147 \pm 0.068$ s.e., z-value = -2.153, p =
270 0.031); we found a close to significant relation for the father's age ($\beta_{father's\ age} = 0.108 \pm 0.058$
271 s.e., z-value = 1.854, p = 0.064).
272

Age of parents and offspring success

274 We found a linear, though close to non-linear, relationship between the father's age and his sons'
275 subsequent breeding success – the sons' yearly reproductive success increased with the father's
276 age (Table 3, Fig. 2; the average yearly breeding success of sons' = 1.89, min = 0, max = 15) but
277 no significant relationship between the father's age and his daughters' reproductive success
278 (Table 3; the average yearly breeding success of daughters' = 1.59, min = 0, max = 9). We did
279 not find any significant link between the mother's age and her sons' or daughters' breeding
280 success (Table 3).
281
282

Discussion

284 Our study showed that the overall male-to-female ratio in this urban population of Blackbirds
285 was not statistically different from 50:50 ratios, so we confirmed our preliminary results and
286 hold the opinion that the results we obtained working on a naturally nesting population of a
287 monogamous species may be the norm in the bird world (Nowacki et al. 2016).
288   Theory predicts that females are expected to increase their overall fitness by adjusting the
289 sex ratio of their broods following their partners' traits. This is possible because male
290 characteristics to some extent approximate the reproductive value of offspring. We found that

291 younger females with older mates tended to produce more sons, probably because of the greater
292 fitness (breeding success) of male descendants. The clutch sequence, nestling hatching sequence
293 and hatching date did not affect the probability of being male.
294   A previous study of our Blackbird population (Jankowiak, Zyskowski & Wysocki, 2018)
295 indicated that "good males" were highly productive from the very beginning of their lives and
296 were more likely to survive to the next year. If lifetime breeding success is highly correlated with
297 the age of birds and old males (>5 years old birds) are the most successful ones, then the optimal
298 strategy for females appears to be to invest in sons when the partner is old. In this case, the age
299 of the partner, or age-related sexually-selected traits in males, could be reliable signals of a bird's
300 quality. Unlike males, the reproductive capacities of females increase with age; hence, if they
301 invest heavily in offspring when young, they will age much faster (Jankowiak, Zyskowski &
302 Wysocki, 2018). We think that older mothers produced more daughters because they were in
303 poorer condition as a result of high early-life fecundity bringing about accelerated senescence
304 (Jankowiak, Zyskowski & Wysocki, 2018). Eggs with male embryos are bigger (Martyka et al.,
305 2010), so the best strategy seems to be to produce smaller eggs with female embryos at a late age
306 (and in worse condition).
307   Reproduction at a late age can also be costly: the transgenerational negative effect of age
308 on sire fitness, known as the "Lansing effect", has been demonstrated in many species in the
309 laboratory (Priest, Mackowiak & Promislow, 2002; Tarín et al., 2005), humans included
310 (Gillespie, Russell & Lummaa, 2013). However, there are few studies of the transgenerational
311 effect in wild populations (Bouwhuis, Vedder & Becker, 2015; Schroeder et al., 2015; Fay et al.,
312 2016). In their study of the House Sparrow *Passer domesticus*, Schroeder et al. (2015) showed
313 that mating with an ageing partner could come at a substantial cost and that the fitness of old
314 fathers was lower. We found, however, that paternal age was almost non-linearly positively
315 related to filial breeding success, which does not falsify the "good genes" hypothesis. We think
316 that a male's advanced age could be a signal of his high quality (the most successful males
317 survive better and live longer (Jankowiak, Zyskowski & Wysocki, 2018); in this population,
318 lifespan is generally closely related to higher lifetime reproductive success (Wysocki et al. 2019,
319 Zyskowski, 2015). The observed slight decline in the breeding success of sons of >6 years old
320 fathers appears to be acting as a trade-off between the costs of senescence and the benefits of
321 "good genes": after the sixth year of life, the costs of paternal senescence outweigh the benefits.
322 Unpublished data concerning our Blackbird population indicate that the number of unhatched
323 eggs in a clutch increases with paternal age, whereas this relation does not hold in females
324 (Jankowiak, Wysocki, personal observations). Hence, the cost of senescence for females paired
325 with old males could be high.
326   The results of studies of the parental effect of age are inconclusive (Booksmythe et al.,
327 2017). In Red-Breasted Flycatcher *Ficedula parva*, females mated to older males produced male-
328 biased clutches (Mitrus, Mitrus & Rutkowski, 2015). In House Sparrow *Passer domesticus*,
329 mothers of intermediate age produced more males, but older females significantly manipulated
330 the sex of their offspring towards daughters (Husby et al., 2006). In a third study, this time of
331 Barn Swallow *Hirundo rustica*, older females produced more daughters (Saino et al., 2002).
332 Similar findings were obtained in the case of a Common Tern *Sterna hirundo* population, in
333 which the more experienced females produced fewer sons (Benito et al., 2013). In Blue Tit
334 *Cyanistes caeruleus* it was found that females produced more sons when, as it turned out, they
335 were mated to males that survived to the next season (Svensson & Nilsson, 1996). A study of
336 Brown Thornbill *Acanthiza pusilla* showed that the offspring proportion was skewed towards

337   sons in broods of pairs with a long-lasting bond (Green, 2002). There are some studies with
338   results contradicting ours, e.g. Blank & Nolan (1983) and Risch & Brinkhof (2002), and in many
339   species researchers failed to find any connection between parental age and sex manipulation (e.g.
340   Bradbury et al., 1997; Sheldon et al., 1999; Leech et al., 2001; Griffith et al., 2003; Dietrich-
341   Bischoff et al., 2006; Delmore et al., 2008; Bowers, Thompson & Sakaluk, 2017). The
342   differential impact of maternal age may be due to differences in breeding biology and the relative
343   contributions of both sexes to raising their progeny. As the contribution on the part of females
344   may increase as they acquire breeding experience early in life, the optimal solution appears to be
345   for older, more experienced females to produce more sons in their "mid-life". But with the onset
346   of senescence, it is more profitable to produce the less costly sex. Because the exact ages of the
347   birds in the papers cited above were not known, their results are equivocal.
348   Relations between male age and the proportion of sons were mostly positive, though non-
349   significant (Booksmythe et al., 2017). But since most authors based their research on two bird
350   age categories (birds in their second calendar year vs older ones), this effect could not have been
351   detected. Only a handful of longitudinal studies have been carried out on wild populations that
352   specified parental age (Saino et al., 2002; Husby et al., 2006; Benito et al., 2013).
353   Another obstacle to understanding the impact of parental age on brood sex ratio could be
354   the lack of information on extra-pair offspring (EPO) in the brood. It is known that females of
355   many socially monogamous bird species often engage in extra-pair copulations (EPC) with males
356   that are more attractive than their social partners (Birkhead & Møller, 1992; Griffith, Owens &
357   Thuman, 2002). This attractiveness, expressed by a male's age/experience or phenotypic traits
358   (body size, ornaments), has a heritable component that a female obtains for her offspring,
359   thereby increasing their fitness (Jennions & Petrie, 2000; Griffith, Owens & Thuman, 2002). To
360   take account of the fact that sons benefit more than daughters from inheriting their fathers'
361   attractiveness traits, females that engage in EPC with more attractive males should bias the sex
362   ratio of EPO towards sons, which is not the case among within-pair offspring (WPO). Most
363   studies have shown a very weak relationship, if any, between EPO and adjustment of the
364   offspring sex ratio (Booksmythe et al., 2017). However, a study of Blue Tits found EPO sex
365   ratios to be son-biased (Kempenaers, Verheyen & Dhondt, 1997). In Coal Tits *Periparus ater*, by
366   contrast, researchers found no evidence for male bias in EPO sex ratios compared to their WPO,
367   but broods without EPO contained relatively more daughters than those with EPO (Dietrich-
368   Bischoff et al., 2006). In this case, the authors indicated that females might not be able to
369   determine which male's sperm had fertilised which egg, but generally manipulated the sex ratio
370   towards sons. EPCs are rare in our population (only 2 out of 98 copulation bouts observed during
371   404 hours of observations during the female fertile period (Wysocki & Halupka, 2004)), but even
372   if EPCs had been more common in our population, it seems to us highly improbable that they
373   would have affected our results. Theoretically, if females engaging in EPC with high-quality
374   males produced more sons, this might suggest that older females with young partners engaged in
375   EPC with high-quality males less often than young females with old males. However, in the light
376   of contemporary knowledge, it is usually older males that obtain more EPO (see Hsu et al., 2015
377   for a review) than younger ones. In the case of females, a study of an Iberian population of Pied
378   Flycatcher *Ficedula hypoleuca* showed that EPP (extra-pair paternity) was higher at nests tended
379   by younger females (Moreno et al., 2015). Older females may be more experienced and
380   dominant, which enables them to avoid extra-pair copulations.
381
382   *Hatching sequence and hatching date*

383 The literature data on hatching sequence and progeny sex ratio appear to be inconclusive
384 (Booksmythe et al., 2017). For instance, a study of Barn Swallows showed that the laying
385 sequence did not affect brood sex manipulation (Saino, Martinelli & Romano, 2008), but in Blue
386 Tits this effect was found to be significant (Cichoń, Dubiec & Stoczko, 2003). In a study seeking
387 to explain the sexual dimorphism of egg size in Blackbirds, Martyka et al. (2010) found that
388 larger eggs contained male embryos but failed to find any relationship between offspring sex and
389 laying order, a fact corroborating our results. Those authors also found that egg size increased
390 with laying order in the clutch, although this applied only to eggs containing female embryos
391 (Martyka et al., 2010). This tallies with our data: we, too, found, albeit without sexing, that later
392 eggs in the clutch were bigger (Cholewa, Jankowiak, Wysocki, unpublished data). The negative
393 effect of being the youngest/smallest in the brood may be greater in daughters because they hatch
394 from smaller eggs, so they incur higher costs of asynchrony than their brothers (Martyka et al.,
395 2010). Therefore, when the mother places more resources in the female last egg in the clutch, she
396 is aiming to increase offspring survival and to cancel out the effect of asynchrony (Clark &
397 Wilson, 1981; Magrath, 1989).
398   We did not find that hatching date and clutch sequence affected the sex ratio in the brood.
399 Seasonal changes in offspring sex ratio have been found in many studies (Graham, Caro &
400 Sockman, 2011; Barclay, 2012): in Barn Swallows (Saino, Martinelli & Romano, 2008), for
401 example, the number of sons in broods increased with laying date, though only in large clutches.
402 These studies confirm that the sex ratio is affected by laying date if the sexes differ with respect
403 to their age of first reproduction or recruitment probability – the sex which needs more time to
404 mature will be produced earlier in the parents' life, while the sex that needs less time to mature
405 hatches later (Dijkstra, Daan & Buker, 1990; Husby et al., 2006; Dijkstra et al., 2010). Studies of
406 Wood Pigeon (*Columba palumbus*) and Rock Dove (*Columba livia*) (Dijkstra et al., 2010)
407 showed that males – in both species this is the sex requiring more time to reach maturity – often
408 hatched earlier in the year, whereas females did so later. Because there was no difference in our
409 Blackbird population between the sexes in the age of first reproduction (GLM with Poisson error
410 distribution, $Chi^2 = 0.005$, $p = 0. 983$, $n_{female} = 700$, female $= 2.47 \pm 0.69$ [mean age $\pm$ s.d.], n_{male}
411 $= 722$, male $= 2.47 \pm 0.68$ s.d.), the mothers should not have preferred either sex. Our results
412 agree with those of the study on Coal Tits (Dietrich-Bischoff et al., 2006), where the brood
413 period had no detectable effect on the sex ratio. We must remember that different species have
414 different biology and ecology and it seems that in blackbirds parental effects could have a
415 stronger impact on offspring sex than changing environmental conditions (e.g. weather, food
416 availability or length of time to improve foraging skills before winter) and females try to
417 maintain the sex ratio at the same level, regardless of the time of the breeding season.
418

Conclusions

420 In our population of Blackbirds, the difference in the parents' age could affect offspring sex, and
421 paternal age could influence offspring fitness. Younger females mated with older males tended to
422 produce more sons because of the greater fitness of male descendants with "good genes" – the
423 sons of older fathers enjoyed a higher breeding success. In contrast, older females mated with
424 younger males produced more daughters: this could have been due to the lesser attractiveness of
425 a younger male and the poorer condition of an old mother.
426

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430
431 **Data Accessibility**
432 Raw data are available here https://github.com/martacho/Offspring-sex-and-fitness-of-
433 blackbirds.git
434

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647

648 **Figure 1.** The results of a fitted model (Table 2) considering only parental age differences; n =
649 382 chicks. The study was performed on Blackbirds (*Turdus merula*) in the Żeromski Park
650 (Szczecin, NW Poland) in 2005-2007 and 2016-2017
651 An age difference of zero signifies the same age of the father and mother, a negative value
652 indicates that the mother was older than the father, while a positive value shows that the father
653 was older than the mother
654
655 **Figure 2.** Relationship between sons' breeding success and paternal age; n = 202. The study was
656 performed on Blackbirds (*Turdus merula*) in the Żeromski Park (Szczecin, NW Poland) in 2006,
657 2007, 2016 and 2017
658

Figure 1

The results of a fitted model (Table 2) considering only parental age differences; n = 382 chicks. The study was performed on Blackbirds (*Turdus merula*) in the Żeromski Park (Szczecin, NW Poland) in 2005-2007 and 2016-2017

An age difference of zero signifies the same age of the father and mother, a negative value indicates that the mother was older than the father, while a positive value shows that the father was older than the mother

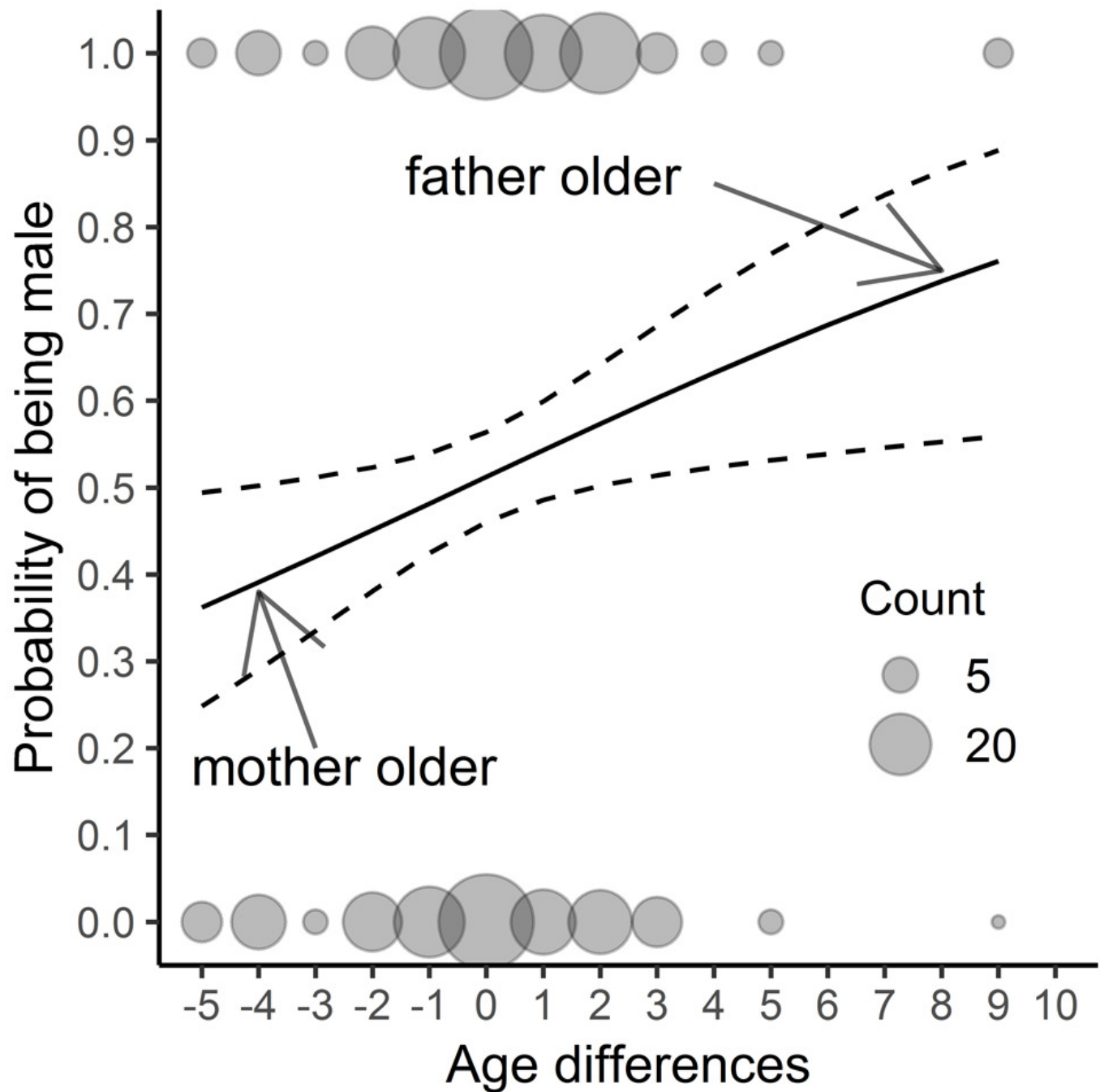
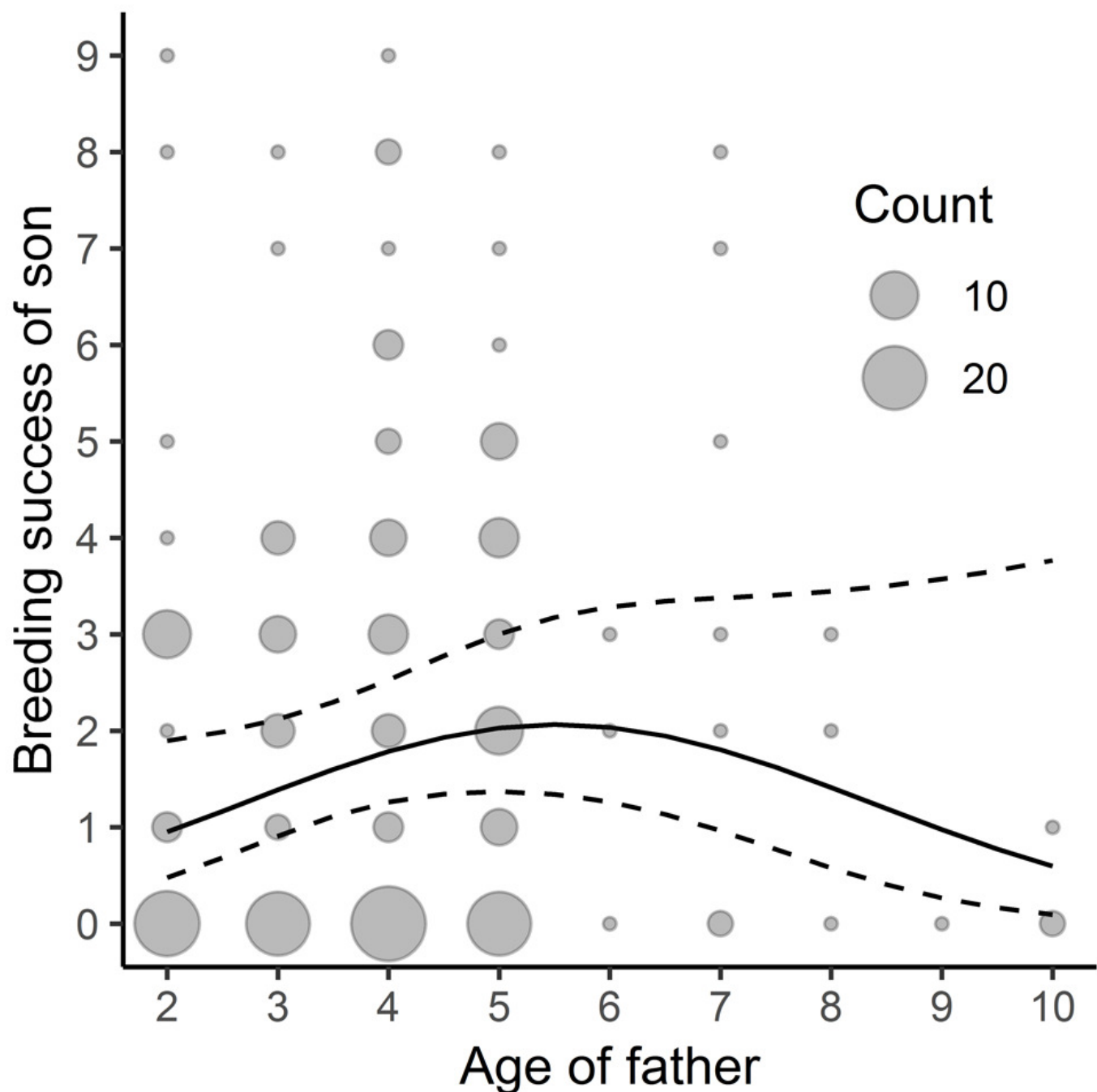



Figure 2

Relationship between sons' breeding success and paternal age; n = 202. The study was performed on Blackbirds (*Turdus merula*) in the Żeromski Park (Szczecin, NW Poland) in 2006, 2007, 2016 and 2017.

Offspring sex ratio (n = 382) according to hatching sequence (Seq) in Blackbirds *Turdus merula*. Data collected in the Żeromski Park (Szczecin, NW Poland) in 2005-2007 and 2016-2017 (overall 126 broods)

1   Table 1. Offspring sex ratio (n = 382) according to hatching sequence (Seq) in Blackbirds *Turdus*
2   *merula*. Data collected in the Żeromski Park (Szczecin, NW Poland) in 2005-2007 and 2016-
3   2017 (overall 126 broods)

Seq	Females	Males	Total
1	52 (43.3%)	68 (56.7%)	120
2	62 (56.9%)	47 (43.1%)	109
3	36 (41.4%)	51 (58.6%)	87
4	30 (55.6%)	24 (44.4%)	54
5	6 (54.5%)	5 (45.5%)	11
6		1	1

The effect of parental age and breeding parameters of natal broods on the probability of male offspring. The study was performed on Blackbirds (*Turdus merula*) in the Żeromski Park (Szczecin, NW Poland) in 2005-2007 and 2016-2017

Estimated parameters of the generalised linear mixed model with binomial error distribution.

Parameters of natal broods: nestling hatching date, clutch sequence in the breeding season,

offspring hatching sequence in the nest; r – random effect

1 Table 2. The effect of parental age and breeding parameters of natal broods on the probability of
2 male offspring. The study was performed on Blackbirds (*Turdus merula*) in the Żeromski Park
3 (Szczecin, NW Poland) in 2005-2007 and 2016-2017

n = 382	Estimate	se/sd	Chi²	P
(Intercept)	-0.330	0.729		
Age difference	0.132	0.052	6.725	**0.009**
Hatching date	-0.003	0.004	0.605	0.437
Clutch sequence	0.099	0.119	0.682	0.408
Offspring hatching sequence	-0.103	0.102	1.023	0.312
r(Nest id)	0.038	0.194		
r(Offspring hatching sequence)	0.023	0.153		

4 Estimated parameters of the generalised linear mixed model with binomial error distribution.
5 Parameters of natal broods: nestling hatching date, clutch sequence in the breeding season,
6 offspring hatching sequence in the nest; r – random effect

The effect of parental age on yearly breeding success of sons and daughters. The study was performed on Blackbirds (*Turdus merula*) in the Żeromski Park (Szczecin, NW Poland) in 2006, 2007, 2016 and 2017

Estimated parameters of a generalised linear mixed model with negative binomial error distribution; yearly breeding success - calculated as the total number of fledglings produced in a given breeding season; id – individual number of nestling, r – random effect. n_{ind} – number of birds (sons/daughters) which bred in the study area; n_{broods} – total number of records used in the analyses; individual birds repeated their breeding attempts in different breeding years

1   Table 3. The effect of parental age on yearly breeding success of sons and daughters. The study
2   was performed on Blackbirds (*Turdus merula*) in the Żeromski Park (Szczecin, NW Poland) in
3   2006, 2007, 2016 and 2017

$n_{ind\ sons} = 75$, $n_{broods} = 202$	Estimate	se/sd	Chi^2	P
(Intercept)	-0.126	0.883		
Father's age	0.687	0.339	4.126	**0.042**
Father's age^2	-0.062	0.033	3.598	0.058
Mother's age	-0.415	0.448	0.863	0.353
Mother's age^2	0.036	0.050	0.533	0.465
r(id)	0.124	0.351		
$n_{ind\ daughters} = 71$, $n_{broods} = 198$				
(Intercept)	-0.606	1.022		
Father's age	0.238	0.337	0.501	0.479
Father's age^2	-0.030	0.036	0.721	0.396
Mother's age	0.315	0.369	0.698	0.403
Mother's age^2	-0.033	0.040	0.635	0.425
r(id)	<0.001	<0.001		

4   Estimated parameters of a generalised linear mixed model with negative binomial error
5   distribution; yearly breeding success - calculated as the total number of fledglings produced in a
6   given breeding season; id – individual number of nestling, r – random effect. n_{ind} – number of
7   birds (sons/daughters) which bred in the study area; n_{broods} – total number of records used in the
8   analyses; individual birds repeated their breeding attempts in different breeding years