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1 **Hatching asynchrony aggravates inbreeding depression in a songbird**

2 **(*Serinus canaria*): an inbreeding-environment interaction**

3 **Raïssa A. de Boer^{1,*}, Marcel Eens¹, Erik Franssen², Wendt Müller¹**

4 ¹ *Faculty of Science - Ethology - University of Antwerp, Universiteitsplein 1 - Campus Drie Eiken C1.25, B-*

5 *2610 Wilrijk, Belgium*

6 ² *StatUa Center for Statistics - University of Antwerp, Prinsstraat 13, B-2000 Antwerp, Belgium*

7 ** Author for correspondence (raïssa.deboer@uantwerpen.be)*

8

9

10 **Abstract**

11 Understanding how the intensity of inbreeding depression is influenced by stressful environmental
12 conditions is an important area of enquiry in various fields of biology. In birds, environmental stress
13 during early development is often related to hatching asynchrony; differences in age, and thus size,
14 impose a gradient in conditions ranging from benign (first hatched chick) to harsh (last hatched chick).
15 Here, we compared the effect of hatching order on growth rate in inbred (parents are full siblings) and
16 outbred (parents are unrelated) canary chicks (*Serinus canaria*). We found that inbreeding depression
17 was more severe under more stressful conditions, being most evident in later hatched chicks. Thus,
18 consideration of inbreeding-environment interactions is of vital importance for our understanding of the
19 biological significance of inbreeding depression and hatching asynchrony. The latter is particularly
20 relevant given that hatching asynchrony is a widespread phenomenon, occurring in many bird species.
21 The exact causes of the observed inbreeding-environment interaction are as yet unknown, but may be
22 related to a decrease in maternal investment in egg contents with laying position (i.e. pre-hatching
23 environment), or to performance of the chicks during sibling competition and/or their resilience to food
24 shortage (i.e. post-hatching environment).

25

26 **Keywords**

27 Maternal effects, growth rate, sibling competition, developmental stress

28

29

30 **Introduction**

31 Inbreeding typically leads to lower fitness of individuals compared to outbreeding (known as
32 inbreeding depression) due to harmful effects of, amongst other things, homozygosity (Charlesworth
33 and Charlesworth 1987; Keller and Waller 2002). Inbreeding depression is, therefore, an important force
34 in evolutionary and ecological processes, and understanding its mechanisms and consequences is an
35 important area of enquiry in biology (Cheptou and Donohue 2011). However, as the expression of genes
36 fluctuates with environmental conditions (“gene-environment interactions”), the effects of inbreeding
37 depression may also vary between environments (“inbreeding-environment interactions”). More
38 specifically, inbreeding depression is thought to be exacerbated under stressful conditions (Armbruster
39 and Reed 2005). This is of particular importance in conservation biology, because environmental
40 deterioration typically relates to population decline and/or fragmentation, which potentially increases
41 the occurrence of inbreeding. An inbreeding-environment interaction in this scenario will lead to an
42 increased risk of extinction (Liao and Reed 2009; Leimu et al. 2010).

43 Previous experimental studies have reported that inbreeding depression increases with
44 environmental stress, for example in studies comparing natural conditions with laboratory conditions; in
45 the case of the latter, environmental variance is supposed to be reduced (Dudash 1990; Chen 1993;
46 Jiménez 1994; Armbruster 2000; Kristensen et al. 2008). However, findings are highly inconsistent across
47 species, populations, and even lineages (Armbruster and Reed 2005). Due to the lack of a generalized
48 pattern, it remains unknown which causal mechanisms underlie inbreeding-environment interactions
49 (Reed et al. 2012). One method to gain more insight into the dynamics of inbreeding depression in
50 relation to the environment is to examine inbreeding depression under varying levels of a natural
51 stressor (Fox and Reed 2011).

52 Here, we propose a new approach that facilitates this type of study in (wild) bird populations.
53 We link inbreeding depression to hatching asynchrony, a natural and widespread stressor that imposes
54 incremental levels of stress. Females lay one egg per day at maximum and vary in the moment at which
55 they initiate incubation. Often incubation starts before the clutch is complete, and hatching of the eggs
56 is consequently spread over multiple days. The position in the hatching order affects, amongst other
57 things, the amount of food allocated by the parents, and is therefore a major determinant of the early
58 environment. The more distant a chick is from the first-hatching position, the more stressful the
59 conditions it will experience (e.g. Stenning 1996; Mock and Parker 1997; Stoleson and Beissinger 1997;
60 Merklings et al. 2014). Hatching asynchrony, thus, imposes a natural gradient in environmental stress
61 within a family lineage.

62 Full-sibling mating of canaries (*Serinus canaria*) was used to generate a group of inbred
63 offspring, while the control group consisted of offspring from pairs of unrelated, outbred birds. Canary
64 chicks hatch asynchronously; we investigated the existence of an inbreeding-environment interaction by
65 comparing the effects of hatching order on growth rate between nests of inbred and outbred offspring.

66

67 **Methods**

68 Study species and housing

69 The canaries originated from an outbred captive population kept at the University of Antwerp.
70 Five weeks prior to breeding, birds were exposed to a long light schedule (14h light : 10h dark). The
71 formation of breeding couples was spread over two clusters; one in February 2013 (93 nests) and the
72 other in April 2013 (37 nests). The additional cluster was required to increase the sample size for an
73 embedded long-term study, and mostly consisted of recombined breeding couples that bred

74 unsuccessfully in the first cluster. Possible differences between the two breeding clusters were
75 accounted for in the statistical analyses.

76 Breeding cages (50 x 64 x 40 cm³, GEHU cages, The Netherlands) were equipped with two
77 perches, shell sand, a nest-cup, nesting material, and constant access to seeds (Van Camp, Belgium) and
78 water. After the first chick hatched, parents were given unlimited access to egg food (Van Camp,
79 Belgium), supplemented with 1 tablespoon/kg Orlux hand mix (Versele-Laga). In addition, freshly
80 germinated seeds were provided daily.

81

82 Experimental design

83 We formed in total 39 nests (cluster 1: 29, cluster 2: 10) with full-sibling parents (inbred
84 offspring), and 91 nests (cluster 1: 64, cluster 2: 27) with unrelated parents (outbred offspring). The
85 formation of more outbred nests is due to the previously mentioned long-term study. Nests were
86 checked daily for hatched chicks from fourteen days (i.e. the minimum incubation period) after the first
87 egg was laid, and hatching success was noted for all nests. For identification purposes, each chick was
88 marked with a non-toxic color marker (Artline 70N) until it was large enough to be fitted with a
89 numbered metal ring. Within all nests, we noted hatching order as 'A' for chicks hatched on day i (71
90 inbred chicks from 38 nests, 160 outbred chicks from 87 nests), 'B' on day $i + 1$ (37 inbred chicks from 28
91 nests, 90 outbred chicks from 72 nests), 'C' on day $i + 2$ (17 inbred chicks from 16 nests, 37 outbred
92 chicks from 33 nests), and 'D' on day $i + 3$ (3 inbred chicks from 3 nests, 9 outbred chicks from 7 nests).
93 As very few chicks from hatching order D were available, the D-hatching order was omitted from the
94 statistical analyses. Chicks that had been cross-fostered after hatching for the purpose of the long-term
95 study were excluded from the growth rate analysis, but taken into account for hatching success. All
96 chicks were weighed daily for 15 days after hatching. At fledging (± 25 days old), a blood sample was

97 taken in order to determine sex; for chicks that did not survive to fledging stage, body tissue was used
98 instead. The ethical committee for animal experimentation at the University of Antwerp approved the
99 above described procedures (file number 2011-86).

100

101 Statistical analysis

102 To study the effects of inbreeding and hatching asynchrony on growth rate, accounting for
103 effects of gender, cluster and viability, we fitted linear mixed models. We took weight (square root
104 transformed to approach normality of the residuals) as the response variable, and included age as an
105 explanatory variable; this is a valid approach, as growth over the time frame studied here is
106 approximately linear (data available from the Dryad Digital Repository:
107 <http://dx.doi.org/10.5061/dryad.tj3pd>). Linear mixed models are robust with regard to the decrease in
108 the number of chicks due to mortality before the end of the study. They are additionally well suited for
109 potential imbalances in sample size for any of the factors (Neter et al. 1996; Bates 2010).

110 For this regression model, multiple observations were performed on the same chick, with
111 several chicks belonging to the same nest. To account for dependency between the observations of the
112 same chick and its nest, we added random-effect terms to the mixed model (Fitzmaurice et al. 2004).
113 Random intercepts were added for nest, and for chick nested within nest, as well as random slope terms
114 for age.

115 The fixed effects of the linear mixed model were: age, genetic condition (inbred or outbred
116 chicks), hatching order (hatching position A , B , or C), cluster (first or second), sex, and viability (survival
117 until day 15 or not). We included 'cluster' as a fixed effect, although it may be considered a random

118 effect; this was to avoid over-complicating the model, and to determine if cluster affected the growth
119 rate of inbred and outbred birds in different ways.

120 The significance of the fixed effects and their interactions was tested using stepwise backward
121 elimination, starting from an initial model that included all fixed effects, their pairwise interactions and
122 the 3-way interactions with age. A significant 3-way interaction with age means that the growth rate is
123 dependent on a combination of 2 factors. The significant 3-way interaction, age*hatching order*genetic
124 condition (see results), was further investigated in 2 ways. First, the dataset was split into 3 subsets
125 according to hatching order; within each of these 3 subsets, we included the interaction term
126 age*genetic condition to test for a difference in growth rate between inbred and outbred chicks.
127 Second, we split the dataset into 2 subsets according to genetic condition; within each of these 2
128 subsets, we included the interaction term age*hatching order to analyze whether the growth rate was
129 different between hatching orders. We created a subset of the full dataset (age = 0) to compare weight
130 at hatching between inbred and outbred chicks with reference to hatching order. For this analysis, only
131 nest identity was included as a random effect.

132 All linear mixed models were fitted using the statistical software package R (R core Team 2014),
133 with the add-on package lme4 (Bates et al. 2014). The F-test with the Kenward-Roger correction was
134 carried out using the 'pbkrtest' package (Halekoh and Højsgaard 2013).

135 The difference in hatching success between inbred and outbred nests was modeled using
136 logistic regression. The experimental unit of this test was the nest, whilst the number of hatched and
137 unhatched eggs within each nest was the response variable. Cluster and genetic condition were the
138 explanatory variables. We tested the significance of genetic condition by performing a likelihood ratio
139 test, comparing the regression models with and without this term.

140

141 Results

142 The mixed modelling revealed a three-way interaction between age, hatching order and genetic
143 condition ($F_{2,317.6} = 5.247, p = 0.0057$); in other words, the difference in growth rate between inbred and
144 outbred chicks depends on hatching order (Figure 1).

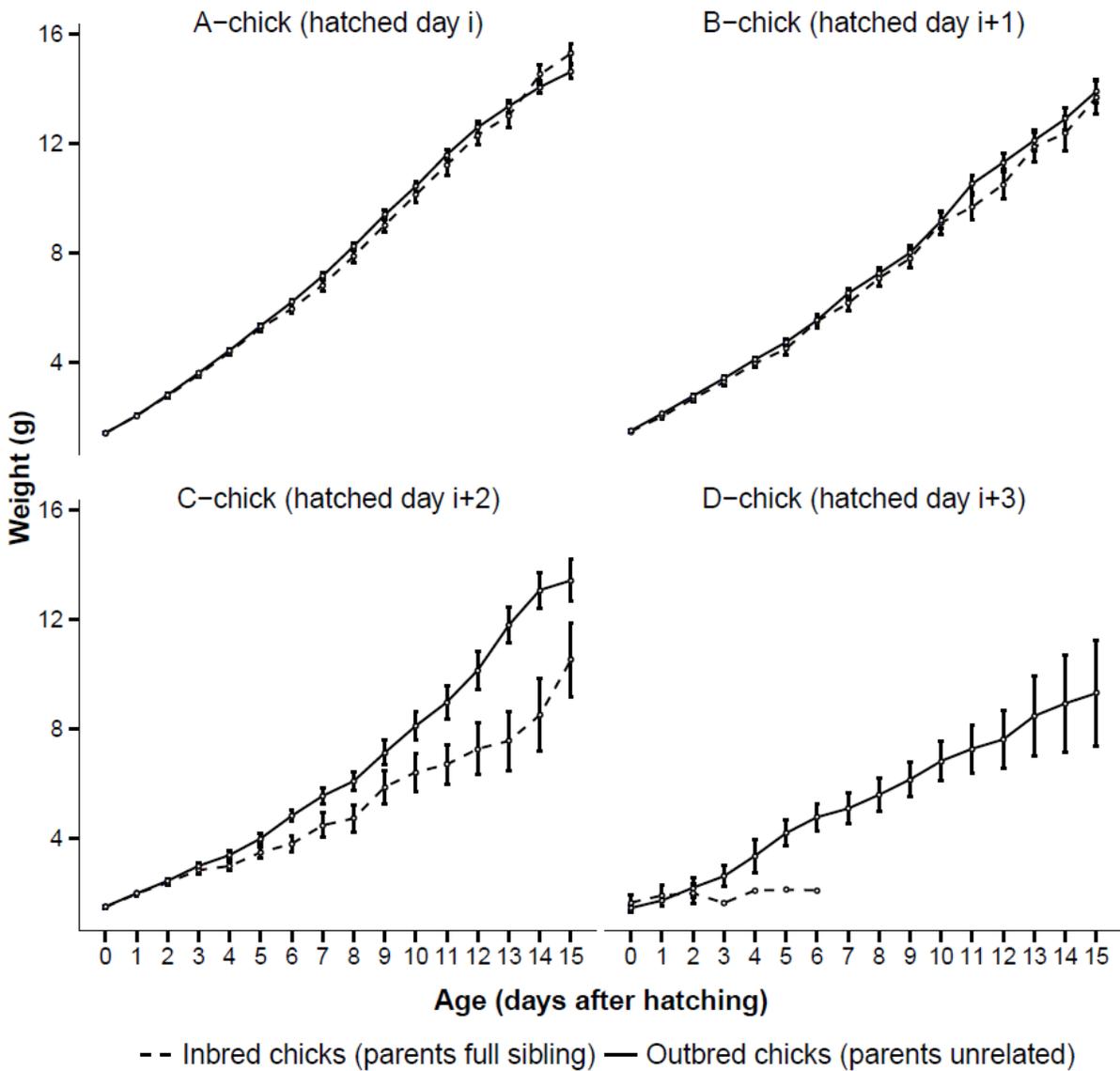
145 Growth rate declined significantly with increasing hatching order (i.e. in later hatching positions)
146 in both inbred ($F_{2,94.8} = 18.161, p < 2 \times 10^{-7}$) and outbred chicks ($F_{2,225.2} = 12.481, p < 7 \times 10^{-6}$). The decline in
147 growth rate for hatching position B (day $i + 1$) when compared to A (day i) was similar in inbred chicks (β
148 = -0.016, SE = 0.0006, $t = -2.54$) and outbred chicks ($\beta = -0.015$, SE = 0.0037, $t = -4.07$). Growth rate was
149 more affected in hatching position C (day $i + 2$) when compared to A (day i) in inbred chicks ($\beta = -0.06$,
150 SE = 0.009, $t = -6.04$) than in outbred chicks ($\beta = -0.02$, SE = 0.006, $t = -3.96$).

151 Splitting the data according to hatching order confirmed that the inbred chicks in hatching
152 position C grew significantly poorly compared to the outbred chicks ($F_{1,39.5} = 8.565, p = 0.005$), while the
153 growth rate did not differ significantly between them in hatching positions A ($F_{1,70.4} = 1.115, p = 0.3$) and
154 B ($F_{1,90.7} = 0.015, p = 0.9$). At the time of hatching, inbred and outbred C-chicks (inbred C-chick age 0:
155 1.48 ± 0.04 g, outbred C-chick age 0: 1.50 ± 0.03 g) were faced with older A-chicks that were almost twice
156 their size (inbred A-chick age 2: 2.75 ± 0.07 g, outbred A-chick age 2: 2.79 ± 0.04 g), as well as larger B-
157 chicks (inbred B-chick age 1: 1.99 ± 0.07 g, outbred B-chick age 1: 2.10 ± 0.04 g). Genetic condition, in
158 interaction with hatching order, did not affect weight at the time of hatching ($F_{3,361.9} = 0.1342, p = 0.3$).

159 Furthermore, we found effects of sex (interaction age*sex: $F_{1,319.4} = 5.683, p = 0.02$), viability
160 (interaction age*viability: $F_{1,409.3} = 88.716, p < 2 \times 10^{-16}$) and cluster (interaction age*cluster: $F_{1,121.4} =$
161 $10.542, p = 0.002$) on growth, but these effects did not differ between inbred and outbred birds (i.e.
162 there were no significant 3-way interactions in: age* genetic condition*sex, age*genetic
163 condition*viability, age*genetic condition*cluster).

164 The probability of survival differed between inbred and outbred chicks; a significantly greater
 165 number of inbred chicks ($30 \pm 1\%$), compared to outbred chicks ($20 \pm 0.6\%$), did not live to 15 days ($\chi^2 =$
 166 126.3 , $df = 1$, $p < 2 * e^{-16}$). Hatching success was significantly lower in inbred nests compared to outbred
 167 nests (inbred = $80 \pm 4\%$, outbred = $90 \pm 2\%$, $n = 130$ nests, $Z_{128,129} = 3.268$, $p = 0.001$).

168



169

170 Figure 1: Growth rate of inbred (dotted lines) and outbred (solid lines) canary chicks from 0 until 15 days
171 after hatching. Growth rate is not affected by inbreeding in the first two hatching positions (A-chicks:
172 top left, n inbred = 71, n outbred = 160; B-chicks: top right, n inbred = 37, n outbred = 90), but in later
173 hatching positions it is hampered in the inbred chicks (C-chicks: bottom left, n inbred = 17, n outbred =
174 37; D-chicks: bottom right, n inbred = 3, n outbred = 9).

175

176 **Discussion**

177 We examined the effects of inbreeding in relation to the (early) environment in nestling
178 canaries, and show that inbreeding depression is exacerbated in stressful environments, represented by
179 a late position in the hatching order.

180 Many bird species hatch their brood asynchronously, because (or in spite) of the gradient in
181 environmental conditions it causes within the nest. Although the exact functional consequences of
182 hatching asynchrony are still subject to debate (reviewed in Stenning 1996), hypotheses are unanimous
183 with regard to the fact that the later a chick hatches, the more stressful the conditions it experiences.
184 Thus, our results support the hypothesis of inbreeding depression being aggravated in harsh
185 environments. The negative effects of hatching asynchrony causing the observed inbreeding-
186 environment interactions may relate to the conditions experienced within the egg (pre-hatching
187 environment), or within the nest (post-hatching environment).

188 Avian females often allocate less resources to later laid eggs, in terms of quality (e.g.
189 antioxidants such as carotenoids and vitamin E; Royle et al. 1999; Blount et al. 2002; Newbrey et al.
190 2014), and quantity (size/weight; Williams 1994; Royle et al. 1999; Nager et al. 2000; Blount et al. 2002).
191 Inbreeding may have caused these chicks to be more susceptible to adverse prenatal conditions

192 compared to those of outbred origin. Unfortunately, we did not measure prenatal maternal resource
193 allocation in this study. Hatching success of clutches laid by full-sibling pairs was indeed lower compared
194 to clutches of unrelated parents; however, this likely relates to an overall reduced viability caused by
195 deleterious effects of inbreeding, as has been previously found in birds (Spottiswoode and Møller 2003;
196 Hemmings et al. 2012).

197 The post-hatching environment of inbred and outbred chicks is strongly related to the food
198 allocation by their parents, which is often not equally distributed over all chicks, with later hatched
199 chicks receiving comparatively smaller portions. This is primarily related to hatching asynchrony (e.g.
200 Mock and Parker 1997; Cotton et al. 1999; Avilés et al. 2011), and this effect can thus also be observed
201 in captivity when food is provided *ad libitum* (Kilner 2002; Müller et al. 2010). Postnatal consequences of
202 a late hatching position may therefore include prolonged periods of starvation or unpredictable access
203 to food. Both aspects increase the nutritional stress experienced by later hatchlings, as for example
204 reflected in increased corticosterone levels (Eraud et al. 2008; Merklings et al. 2014) and reduced growth
205 (e.g. this study). Inbreeding may render later hatched chicks less resilient against these environmental
206 challenges.

207 Alternatively, inbreeding may have weakened the performance of these chicks in sibling
208 competition, since hampered food acquisition in later hatching positions is also due to a decreased
209 competitive potential (Mock and Parker 1997; Viñuela 1999; Nathan et al. 2001). Indeed, studies in
210 plants and invertebrates, even as early as Darwin's work, have shown that inbreeding depression is
211 more notable when the level of competition is high (e.g. Darwin 1876, Carr and Dudash 1995; Bijlsma et
212 al. 1999; Cheptou et al. 2000; Cheptou et al. 2001; Valtonen et al. 2014). The degree to which an
213 individual is able to perform in competition involves many physiological processes, arguably also
214 increasing the chance of exposing inbreeding depression in vertebrates (Meagher et al. 2000; Keller et al

215 2002; Gallardo and Neira 2005). Therefore, increased stress in the late hatching positions, caused by
216 sibling competition, may explain our finding of hampered growth in late hatched inbred chicks.

217 Our study is, to the best of our knowledge, the first to use hatching asynchrony with the
218 purpose of studying inbreeding-environment interactions, while hatching asynchrony in itself is a well-
219 studied phenomenon. The results are in line with a number of previous studies on (wild) bird
220 populations which have shown that inbreeding depression increases under environmental stress (Keller
221 et al. 2002; Marr et al. 2006; Szulkin and Sheldon 2007). These studies mainly used environmental
222 parameters (e.g. rain, temperature) to quantify the level of environmental stress. However, the exact
223 effect at the level of an individual remains unknown, although the response to stressful situations may
224 vary within and between lineages (Dudash 1990; Fox and Reed 2011). We propose to use hatching
225 asynchrony as an axis of environmental stress, as it enables the examination of inbreeding-environment
226 interactions within a family lineage (i.e. a nest). In fact, whenever data on relatedness and hatching
227 asynchrony are available from studies on wild bird populations, our approach can be taken.

228 Finally, hatching asynchrony has the advantage that it creates a natural gradation in the
229 parameter 'stress', that ranges from benign for the first hatched chick to intense for the last hatched
230 chick. This minimizes the chances that a manipulation imposes either too much or too little stress, which
231 has previously been argued to be one explanation for contradicting results on this subject (Armbruster
232 and Reed 2005; Fox and Reed 2011).

233 The importance of implying variance in stress is clearly demonstrated in our study because
234 growth did not differ in the first two (out of four) least stressful conditions (hatching orders A and B),
235 but the subtle increase in stress in hatching order C had a substantial effect on inbred chicks.

236 Thus, hatching asynchrony in combination with inbreeding is a fruitful approach to the study of
237 inbreeding-environment interactions. In the future, we aim to unravel the exact mechanism(s)

238 underlying the differential response to a later hatching position between inbred and outbred birds. We
239 anticipate that inbreeding will mainly affect the ability to compete for food, and will, thus, lead to
240 increased inbreeding depression under more stressful, more competitive conditions.

241

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249

250 **Data archiving**

251 <http://dx.doi.org/10.5061/dryad.tj3pd>

252

253 **Short title**

254 Hatching order affects inbreeding depression

255

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