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MASTER'S THESIS

**“MHC supertypes affect survival and lifetime reproductive success in a population of pied flycatchers”**

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## 1 **Abstract**

2 The major histocompatibility complex (MHC) plays an essential role in the immune response  
3 against pathogens. Their high polymorphism is thought to be mainly the consequence of host-  
4 pathogen coevolution, but demonstrating the underlying mechanisms driving MHC evolution  
5 remains challenging in natural populations. We investigated in a wild population of pied  
6 flycatchers *Ficedula hypoleuca* the effect of MHC class II functional diversity, supertypes, on  
7 two key aspects of individuals' fitness: lifetime reproductive success and survival. In  
8 particular, we tested four hypotheses about pathogen-mediated selection on MHC: (i)  
9 *maximum diversity hypothesis*, which states that fitness increases as the number of supertypes  
10 increases; (ii) *optimal diversity hypothesis*, which states that an intermediate number of  
11 supertypes confers the highest fitness; (iii) *fluctuating selection hypothesis*, which states that  
12 some specific supertype, unrelated to its frequency in the population, affect fitness; and (iv)  
13 *rare-supertype advantage hypothesis*, which states that rare supertypes provide fitness  
14 benefits where common ones entail costs for the individuals. We found that estimated effects  
15 of each supertype on lifetime reproductive success were negatively related to their frequency  
16 in the population. A similar association for the estimated effects of each supertype was found  
17 on apparent survival, but it was not statistically significant. Individuals with supertypes 11  
18 and 12, which were among the most common in the population, had lower survival and  
19 lifetime reproductive success. In contrast, those with supertype 7, rare in the population,  
20 attained higher lifetime reproductive success. Overall, our findings suggest that *rare-*  
21 *supertype advantage hypothesis* may be at work in this population, with the emergence of  
22 new supertypes that are positively selected until pathogens evolve resistance and protective  
23 advantage decreases or disappears.

24 **Keywords:** lifetime reproductive success, major histocompatibility complex (MHC), mark-  
25 capture-recapture models, MHC supertype, pied flycatchers, survival.

## 26 **Introduction**

27 Understanding the genetic basis of fitness differences is a central issue in evolutionary  
28 biology (Ellegren and Sheldon 2008). Major histocompatibility complex (MHC) is a group of  
29 genes, widely found in vertebrates (Bernatchez and Landry 2003), that plays an essential role  
30 in innate and adaptive response immunity (Rees and Mian 1999, Gavlovsky et al. 2016). The  
31 molecules produced by these genes present foreign antigens from viruses, bacteria or parasites  
32 to T lymphocytes initiating the cascade of immune responses (Trowsdale 1993). In particular,  
33 MHC Class II has a second exon that has received much attention, encompassing a wide  
34 range of wild animals (fishes: Wegner et al. 2003b, Šimková et al. 2006; amphibians: Bataille  
35 et al. 2015; birds: Agudo et al. 2012, Pineaux et al. 2020; mammals: Aguilar et al. 2004, Li et  
36 al. 2011). Although its involvement in resistance or susceptibility to different pathogens is  
37 widely studied, there is still controversy on the exact mechanism underlying MHC evolution  
38 and how MHC variation influences the fitness of individuals in wild populations (Radwan et  
39 al. 2020).

40 MHC is the most polymorphic gene group known in vertebrates. While other  
41 mechanisms may be involved (Hedrick and Thomson 1988, Potts et al. 1994, Hedrick 1999),  
42 balancing selection driven by host-pathogen coevolution seems to be the main reason for  
43 MHC extreme polymorphism (Wegner et al. 2003b, Borghans et al. 2004). Three, non-  
44 mutually exclusive (Westerdahl et al. 2005), mechanisms of pathogen-mediated selection  
45 have been proposed for maintaining MHC diversity (reviewed in Spurgin and Richardson  
46 2010). First, because individuals must deal with a wide variety of pathogens, heterozygous  
47 individuals or those with high allelic diversity may have an advantage in developing MHC  
48 molecules capable of targeting a wide range of infectious diseases (Doherty and Zinkernagel  
49 1975, Oliver et al. 2009). Under this rationale, two hypotheses have been proposed: the  
50 *maximum diversity hypothesis* stating that fitness increases with MHC diversity and the

51 *optimal diversity hypothesis* stating that intermediate MHC diversity confers the highest  
52 fitness (Wegner et al. 2003a, Bonneaud et al. 2004, Madsen and Ujvari 2006, Thoß et al.  
53 2011). The rationale behind the *optimal diversity hypothesis* is that a high MHC diversity may  
54 be inefficient or even detrimental (Woelfing et al. 2009), as the number of different MHC  
55 molecules expressed during the immune response is limited (Nowak et al. 1992). Second, the  
56 *fluctuating selection hypothesis* states that MHC diversity is influenced by environmental  
57 changes that cause spatio-temporal variation of pathogens (e.g. between different habitats or  
58 according to climatic factors). Thus, MHC diversity would be maintained by directional  
59 selection of different specific MHC alleles at different times and/or sites (Hedrick 2002,  
60 Jensen et al. 2008, Blanford et al. 2013). Third, under the *rare-supertype advantage*  
61 *hypothesis*, specific MHC alleles are necessary to cope with pathogens (Sepil et al. 2012,  
62 Karlsson et al. 2015), but their selection would respond, unlike the previous mechanism, to  
63 their population frequency (Hedrick 1999). Thus, individuals carrying a new allele, efficient  
64 against some pathogens, would enjoy an advantage over the others (Bodmer 1972, Borghans  
65 et al. 2004). As a result, that allele frequency in the population would increase until the allele  
66 is no longer advantageous and the coevolution process would favour the emergence of other  
67 new rare alleles.

68         Several studies have investigated the effects of allelic diversity, as a measure of MHC  
69 diversity, on different proxies of fitness (reviewed in Bernatchez and Landry 2003). However,  
70 allelic diversity does not appear to be a good indicator of the ability of individuals to cope  
71 with multiple pathogens. Individuals with high allelic diversity may possess very similar  
72 alleles, while those with low allelic diversity may possess sufficiently divergent alleles to  
73 respond to different potential pathogens (Pierini and Lenz 2018). Thus, functional diversity  
74 both within loci (degree of heterozygosity) and between loci may be important (Schwensow  
75 et al. 2019). Therefore, a more efficient approach to study the consequences of MHC diversity

76 on the immune response is the clustering of alleles into supertypes (Naugler and Liwski  
77 2008), according to the physicochemical properties of the amino acids involved in antigen  
78 binding (Sette and Sidney 1998, Reche and Reinherz 2007). This is because the existence of  
79 some specific supertype or a large number of supertypes (rather than alleles) may confer an  
80 advantage over individuals whose allele combinations do not form such clusters (Sepil et al.  
81 2012, 2013, Kubinak et al. 2012, Lighten et al. 2017).

82 Most previous works on MHC have focused on its effects on some proxy of fitness  
83 (Bernatchez and Landry 2003). However, MHC composition may have differential effects on  
84 fitness components (e.g. Worley et al. 2010, Thoß et al. 2011), such that focusing on a single  
85 fitness component may lead to incorrect conclusions (Siepielski et al. 2011, Huang et al.  
86 2020). As individuals may follow different life-history strategies (Pigeault et al. 2018), it is  
87 critical to jointly investigating the effects of MHC on different components of survival (Penn  
88 et al. 2002, Westerdahl et al. 2005, Šimková et al. 2006, Brouwer et al. 2010) and  
89 reproduction (Kalbe et al. 2009, Thoß et al. 2011, Sepil et al. 2013). This is challenging in  
90 natural populations because it requires individual-based long-term studies on populations with  
91 high fidelity rates.

92 This study used an individual-based, long-term dataset from a population of pied  
93 flycatchers *Ficedula hypoleuca* to investigate whether quantitative or qualitative aspects of  
94 MHC class II supertypes affect individual fitness, measured as both lifetime reproductive  
95 success (LRS) and survival. Pied flycatchers, including our study population, show high  
96 breeding-site fidelity (Lundberg and Alatalo 1992, Canal et al. 2011, Camacho et al. 2019,  
97 Potti et al. 2021), which allow monitoring of individuals throughout their life and make the  
98 species an ideal system to study MHC evolution. We aimed to test the following prediction of  
99 four hypotheses associating MHC and fitness: (i) *maximum diversity hypothesis*, fitness  
100 increases as MHC diversity increases; (ii) *optimal diversity hypothesis*, an intermediate MHC

101 diversity confers the highest fitness; (iii) *fluctuating selection hypothesis*, the effect on fitness  
102 vary between specific supertypes, but with no relation to their frequency in the population;  
103 (iv) *rare-supertype advantage hypothesis*, the effect of fitness vary between specific  
104 supertypes and is negatively associated to their population frequency.

105

## 106 **Materials and Methods**

### 107 **Study system**

108 We investigated MHC-individual fitness associations using data obtained from as a part of a  
109 long-term study of 162 pied flycatchers (85 males and 77 females) from a natural population  
110 breeding in nest-boxes in La Hiruela, Central Spain (ca. 41° 4' 42" N, 3° 25' 55" W, 1200 –  
111 1300 m a.s.l.). The study area consists of two plots, a natural oak forest (*Quercus pirenaica*)  
112 and a conifer plantation dominated by *Pinus sylvestris*, separated by 1.1 km. There are 237  
113 nest-boxes, at a mean distance of 20 m from each other (Camacho et al. 2019). Blood samples  
114 for MHC genotyping were obtained from adults during two consecutive breeding seasons  
115 (2005 and 2006). All sampled individuals had born in the nest-boxes, so their exact age is  
116 known. The recapture (breeding) histories of these sampled individuals span from 2000 to  
117 2011.

118

### 119 **Field work and general procedures**

120 Field protocols have been described in detail elsewhere (Canal et al. 2011, Camacho et al.  
121 2013). Briefly, during the breeding season, which lasts from around the third week of April  
122 (when first males arrive from migration) to the first fortnight of July, all nests were regularly  
123 checked to ascertain laying date, clutch size (typically, 5-6 eggs), hatching date and number of

124 fledglings. Parent birds were captured while incubating (females) or with a nest-box trap  
125 while feeding eight-day-old nestlings (males and females). They were weighed, measured at  
126 standard morphological traits and individually marked with a numbered metal band and a  
127 unique combination of coloured bands (only males). Due to the strong breeding fidelity of  
128 adults in the study population (Potti and Montalvo 1991c, Potti et al. 2013, Canal et al. 2014),  
129 the exact age of many birds was known (53% of adults were born in the nest-boxes; Potti and  
130 Montalvo 1991c). Unringed breeders were aged as first-year or older based on plumage traits  
131 following ageing criteria described in Karlsson et al. (1986) and Potti and Montalvo (1991c).  
132 Blood samples were taken from all individuals by puncturing the brachial vein and stored in  
133 absolute ethanol.

134 Fledglings were banded, measured and weighed at 13 days of age. This allowed us to  
135 establish their fate (whether they recruited or not) in the following years and assess for each  
136 breeding adult their lifetime reproductive success. In particular, individuals show strong natal  
137 site fidelity, with up to 22% of the fledglings returning to the natal site, this being among the  
138 highest rates reported for the species (Potti and Montalvo 1991a, Canal et al. 2014). Further,  
139 there is no familial resemblance in dispersal patterns. Thus, offspring of individuals with a  
140 high propensity to stay/disperse are not more prone to stay/disperse (Camacho et al. 2015).  
141 Finally, breeding outside the study plots, either in the surroundings (as indicated by surveys  
142 conducted during the breeding seasons) or in more distant areas, including other study  
143 populations of Iberian flycatchers (as indicated by ring recoveries), is an extremely rare event  
144 (pers. obs.; Potti and Montalvo 1991b). This suggests that most surviving fledglings  
145 reproduce in the study population. Therefore, it is reasonable to assume that the recruits in the  
146 population are an unbiased sample of all recruits.

147

148 **MHC genotyping**

149 DNA was extracted using the Qiagen blood extraction kit. We used the primers MHCIIFiHy-  
150 E2CF and MHCIIFiHy-E2CR, located in the introns-exon 2 junction, to amplify the entire  
151 coding region of exon 2 (270 pb) across a multigene family of functionally relevant MHC  
152 class II B genes in pied flycatchers (see Canal et al. (2010) for details).

153 Bidirectional pyrosequencing was performed in a Roche/454/ GS-FLX pyrosequencer.  
154 Previously to pyrosequencing, each individual was marked with the GS Junior Titanium  
155 Fusion Primers, a universal tail (provided by Roche) and a MID (Multiplex identifiers) of 10  
156 pb which allows assigning sequences within individuals (structure of primers: 5'- GS Junior  
157 Titanium Fusion Primers -Tag - Universal tail - Primers *Ficedula hypoleuca* - amplicons -3').  
158 PCRs were performed in 25- $\mu$ L reaction volumes containing 1x manufacturer-supplied buffer  
159 (Bioline), 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP, 1 U Taq Polymerasa, 0.2  $\mu$ M of each  
160 primer, 5% Dimethyl sulfoxide (DMSO), 10  $\mu$ g of BSA (Bovine Serum Albumin - Amersham  
161 corp.), and 25 ng of DNA as a template. PCR amplifications consisted of initial denaturation  
162 of 2 min at 94°C, followed by 23 cycles of 30 s at 94°C, 30 s at an annealing temperature of  
163 56°C and 40 s at 72°C, plus a final extension of 10 min at 72°C. We ran PCRs with a reduced  
164 number of cycles and long elongation times as this has been suggested to diminish the risk of  
165 chimaeras formation (Lenz and Becker 2008). To obtain an accurate quantification of the  
166 PCR products, ADN quantification was done by fluorometry using the Quant-iT Picogreen  
167 dsDNA Assay kit (Invitrogen, San Diego, CA). Amplicons were pooled in approximately  
168 equimolar quantities, purified using AMPure beads, amplified by emulsion-based clonal  
169 amplification (emPCR) and sequenced. Purification, amplification and sequencing were  
170 performed following protocols provided by Roche.

171           When high-throughput sequencing technologies are applied, it is common to obtain a  
172 high number of artefacts. Thus, we followed a stepwise procedure to distinguish real alleles  
173 from artefacts. First, we excluded individuals with low coverage (<200). Second, the software  
174 GENEIOUS (Kearse et al. 2012) was used to filter out chimaeras originated by in vitro  
175 recombination between true alleles (Lenz and Becker 2008, Galan et al. 2010). To this end,  
176 when grouped the sequences within individuals by similarity, thus chimaeras could be easily  
177 detected because they typically appear in a low number between the two clusters of sequences  
178 corresponding to the putative alleles from which they are originated. Third, we translated the  
179 sequences to verify that they were functional (e.g. without stop codons) and checked visually  
180 that variants were located in polymorphic sites. When nucleotide sequences differed due to  
181 synonymous substitutions, they were considered the same alleles. Finally, we only considered  
182 a sequence as an allele when it was present in at least 2 different individuals. Following these  
183 criteria, we identified 97 alleles in the population.

184           As a control, 12 individuals were genotyped by duplicated (i.e. they were amplified in  
185 independent PCRs, sequenced with different barcodes and blindly analyzed) and found no  
186 discrepancy between their genotypes. Further, coverage and number of alleles found in an  
187 individual were unrelated (Pearson correlation:  $r = 0.11$ ,  $p = 0.12$ ), suggesting that our  
188 genotyping approach is reliable. However, it is possible that, despite following all the steps  
189 above, artefacts were not completely removed. In such a case, it is reasonable to assume that  
190 individuals with higher reproductive success or survival rates are not more likely to generate  
191 artefacts than other individuals. Thus, it is unlikely that artefacts bias our conclusions.

192           In addition to the number of alleles, we also estimated the number of supertypes of  
193 each individual. Clustering was carried out following the methods described by Doytchinova  
194 and Flower (2005). Briefly, regions corresponding to antigen binding sites (ABS) were  
195 identified, which are coding sequences characterized by being under positive selection

196 resulting in excess of nonsynonymous substitutions (dN) over synonymous substitutions (dS).  
197 dN/dS ratios were calculated in MEGA 4.1 using a Modified Nei-Gojobori method with  
198 Jukes-Cantor correction and statistically supported with Z tests run in MEGA 4.1. After  
199 identifying the positively selected sites, each of its amino acids was characterized by five  
200 physicochemical descriptor variables: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4  
201 and z5 (electronic effects). The data matrix obtained was subjected to k-means clustering  
202 analysis using the "find.clusters" function in software R version 4.4.0 ([http://www.R-](http://www.R-project.org)  
203 [project.org](http://www.R-project.org)). Different clustering solutions were compared by Akaike's Information Criterion  
204 (AIC; Akaike, 1987), and the most parsimonious solution was selected. Ultimately, 14  
205 supertypes were identified in our study population.

206 We excluded data from those individuals involved in experiments (e.g., cross-  
207 fostering) conducted during the long-term study with potential effects on reproductive success  
208 or survival. Further, as field effort was reduced in 2003, we performed the analyses excluding  
209 individuals breeding in that year. Finally, the associations between MHC class II and fitness  
210 components were assessed in 99 breeding adults (52 females and 47 males).

211

### 212 **Does LRS depend on MHC overall diversity and/or specific supertypes?**

213 We used generalized linear mixed models (GLMM) to investigate the effect of MHC  
214 genotype on lifetime reproductive success (response variable), measured as the number of  
215 fledglings produced over the individual lifetime. All the models included the additive effects  
216 of "habitat" (two-class levels: conifer plantation and oak forest) and "sex" as controlling  
217 predictors and "birth year" (seven class levels: 1999-2005) as a random intercept. Before  
218 fitting the models, we compared using the corrected Akaike Information Criterion corrected  
219 for small samples (AICc; Burnham et al. 2011) the goodness-of-fit of several candidate

220 models using different error distributions (e.g. Poisson and Negative Binomial). That analysis  
221 showed that the most compatible model had a Conway-Maxwell-Poisson distribution (data  
222 not shown).

223 First, we evaluated the *maximum* and *optimal diversity hypotheses* by fitting a model  
224 with, respectively, the linear and quadratic effect of the total number of supertypes per  
225 individual. Second, we evaluated the influence of specific MHC supertypes on lifetime  
226 reproductive success, setting as focal predictor the presence/absence of each of the 14  
227 supertypes described in our study population. By doing so, we tested whether, as expected by  
228 both *fluctuating selection* and *rare-supertype advantage hypotheses*, the presence of a specific  
229 supertype affected lifetime reproductive success. In total, we fitted 16 models (Table 1).

230 We compared the AICc (Burnham et al. 2011) value of the different models with the  
231 null model to rank the models. Further, we calculated the standard error and the p-value using  
232 the Likelihood Ratio Test between each model and the null model. Given that we conducted  
233 multiple tests under the same hypothesis, we applied the Benjamini-Yekutielle correction  
234 method (Benjamini and Hochberg 1995) as detailed in Pike (2011). As a result, the  
235 significance level dropped from 0.05 to  $p < 0.0148$ .

236 Before interpreting any model, we systematically performed several diagnostic  
237 statistics (e.g., distribution of residuals, the existence of multicollinearity) to avoid misleading  
238 results due to statistical artefacts. These statistics did not indicate obvious deviation from the  
239 assumptions of linear models. Analyses were performed in software R version 4.4.0 using the  
240 package “glmmTMB” (Brooks et al. 2017). The packages “DHARMA” (Hartig 2021) and car  
241 (VIF function) were used for model diagnostics, and the plots for each predictor’s effect were  
242 obtained with the “effects” (Fox and Weisberg 2019) and “ggplot2” (Wickham 2016)  
243 packages.

244 **Does apparent survival depend on MHC overall diversity and/or specific supertypes?**

245 *Multievent capture-recapture modelling*

246 To estimate apparent survival ( $\Phi$ ) and ( $p$ ) resighting probabilities, we used capture-mark-  
247 recapture Cormack-Jolly-Seber (CJS) in program E-SURGE version 2.2.3 (Choquet et al.  
248 2009b). In E-SURGE, a distinction is made between what is observed in the field (events) and  
249 the underlying biological states because when they do not match, a multievent (Pradel 2005)  
250 modelling approach allows for the incorporation of uncertainty (incomplete or misclassified  
251 data) in event assignment. We considered two states: (i) live individual, (ii) dead individual,  
252 and two events with the corresponding number used in the data set: (i) not seen – “0”, (ii)  
253 seen – “1”.

254

255 *Goodness of Fit*

256 Previously to the capture-mark-recapture analysis, goodness of fit tests (GOF) were run in U-  
257 CARE version 3.3.0 (Choquet et al. 2009a) to determine if the CJS model fits our data. In this  
258 study, we do not consider age classes. GOF tests allow to determine whether there is a lack of  
259 fit and, in many cases, identify the causes. First, the transient effect (see Test 3.SR in U-  
260 CARE) indicates that the apparent survival probability is different in the first interval than in  
261 subsequent intervals. A transient effect reveals some heterogeneity in the survival/fidelity  
262 process of the sampled individuals, which may be the result of age-specific survival, the  
263 temporal presence of some individuals in the study area (e.g., presence of prospecting  
264 individuals) or an increase in mortality immediately after the physical handling at marking.  
265 Second, trap-dependence (Test 2.CT) occurs when the probability of resighting in one session  
266 (breeding season in our case) depends on the resighting event in the previous one. Trap-

267 dependence is expected when the capture process affects individuals' behaviour, making them  
268 more (trap happiness) or less (trap shyness) likely to be captured in the subsequent session.

269 The global test was not significant ( $\chi^2 = 0.5666$ , d.f. = 6,  $p = 0.99693$ ), meaning that the  
270 CJS model fits the overall data well. We did not find evidence of transient-effect ( $Z = -$   
271  $0.15779$ ;  $p = 0.56269$ ) or trap-dependence ( $Z = 1.6697e^{-15}$ ;  $p = 1$ ).

272

### 273 *Model selection on capture-recapture models*

274 We first modelled the entire dataset, independent of the MHC genotype of individuals. We  
275 considered four covariates in the global (most parameterized) model: (i) birth year (between  
276 1999 and 2005); (ii) sex; (iii) habitat (conifer plantation or oak forest); (iiii) age at first  
277 reproduction (one-year-old or >1yo). Following a common notation for capture-mark-  
278 recapture models (Lebreton et al. 1992), our global model can be expressed as:  $\Phi$  (birthyear +  
279 sex\*habitat\*agereprod1)  $p$  (time + sex + habitat), where "\*" and "+" are, respectively, non-  
280 additive and additive relationships. So, apparent survival depended on the additive effect of  
281 the birth year of individuals and the interaction between sex, habitat and age at first  
282 reproduction. In contrast, the resighting depended on the additive effects of sex, habitat and  
283 time. This global model was our starting model for subsequent model selection (Burnham and  
284 Anderson 2002).

285 The rank of different models was based on AICc (Burnham et al. 2011) to determine  
286 the relative support of each one according to the principle of parsimony. Our model selection  
287 followed two steps. In step one, we compared the AICc of models nested to the global model  
288 in both resighting and survival probability. Following a standard procedure (Lebreton et al.  
289 1992), we started modelling the resighting probability, leaving the survival probability fixed  
290 as in the global model [ $\Phi$  (birthyear + sex\*habitat\*agereprod1)]. The resulting most

291 parsimonious model (lowest AICc) included the single effect of sex on the resighting  
292 probability. Then, we repeated the process with the survival probability (holding [ $p$  (sex)])  
293 finding the most support for the model  $\Phi$  (birthyear + agereprod1).

294 In step two, we started from the so far most parsimonious model [ $\Phi$  (birthyear +  
295 agereprod1)  $p$  (sex)], to test the effect of genetic variables on the apparent survival. The  
296 variables included in the survival analyses were similar to those considered in the lifetime  
297 reproductive success analyses (see above). In total, we run 16 models (Table 3) considering  
298 the following set of variables: (i) To test the *maximum* and *optimal diversity hypotheses* we  
299 used the standardized number of supertypes of each individual. (ii) We evaluated the effect of  
300 each supertype on apparent survival to test the *fluctuating selection* and *rare-supertype*  
301 *advantage hypotheses*.

302 We reported the effect size (95% CI) of each genetic trait on the apparent survival. For  
303 a correct fit and estimation of the models by maximum likelihood and to achieve the correct  
304 global minima, the models were run with three random initial values (option multiple random  
305 in E-SURGE; (Choquet and Nogué 2011)). In this study, as the time between each sampling  
306 was unequal (no sampling in 2003), setting a file of unequal time intervals was necessary to  
307 accurately estimate apparent survival (Choquet and Nogué 2011). Subsequently, graphical  
308 figures corresponding to the effect sizes and survival rates of top models were obtained in  
309 software R version 4.4.0 using the “ggplot2” package (Wickham 2016).

310

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313

314 **Does the effect of specific supertypes on LRS and apparent survival correlate with their**  
315 **population frequency?**

316 We tested whether the effects of each supertype were related with its population frequency in  
317 order to elucidate whether selection on specific supertypes was determined by the *fluctuating*  
318 *selection hypothesis* or the *rare-supertype advantage hypothesis*.

319 For that aim, we used linear regressions with, as the predictor, the supertype frequency  
320 among the sampled individuals (Figure 1) and, as the response variable, the estimated effects  
321 of each supertype on LRS and apparent survival as estimated by our previous analyses.  
322 Graphical figures for each linear regression were obtained with the “effects” (Fox and  
323 Weisberg 2019) and “ggplot2” (Wickham 2016) packages.

324

325 **Results**

326 **Does LRS depend on MHC overall diversity and/or specific supertypes?**

327 Contrary to what is expected by the *maximum* and *optimal diversity hypotheses*, we found no  
328 effect of MHC class II diversity, both linear and quadratic (Table 1, models 3 and 7 vs null  
329 model) on lifetime reproductive success. By contrast, the lifetime reproductive success of  
330 individuals was related to the presence of three specific supertypes: 12, 11 and 7 (Table 1,  
331 models 1, 2 and 3 vs null model). Individuals with supertype 12 (Figure 2a;  $\beta = -0.346$ , SE =  
332 0.1, p-value = 0.0007) or supertype 11 (Figure 2b;  $\beta = -0.435$ , SE = 0.13, p-value = 0.0013)  
333 have a lower number of fledglings over their lifetime than individuals without those  
334 supertypes. However, supertype 7 has the opposite effect, i.e., individuals with supertype 7  
335 have higher lifetime number of fledglings than those without supertype 7 (Figure 2c;  $\beta =$   
336 0.632, SE = 0.23, p-value = 0.0107).

337 **Does apparent survival depend on MHC overall diversity and/or specific supertypes?**

338 We found no support for the *maximum* and *optimal diversity hypothesis* predictions that  
339 individual survival depends, linearly and quadratically, on the MHC class II diversity (Table  
340 2, models 11 and 21 vs base model). However, we found clear support for the effect of  
341 supertype 12 on pied flycatcher survival rates (Table 2, model 1 vs base model). On average,  
342 the individuals carrying supertype 12 had a lower probability of survival ( $\beta = -1.048$ , SE =  
343 0.357) than those without supertype 12 (Figure 3). The model was the best supported in the  
344 set of candidate models (Table 2), and the 95% estimates of the coefficient did not include  
345 zero (Figure 4).

346 Also, the models testing the effect of supertype 7 and supertype 11 showed a lower  
347 AICc than the null model (Table 3, models 2 and 3 vs base model), but their 95% estimates of  
348 the coefficient included zero (Figure 4).

349

350 **Does the effect of specific supertypes on LRS and apparent survival correlate with their**  
351 **population frequency?**

352 In agreement with the *rare-supertype advantage hypothesis*, we found a negative relationship  
353 between the effects of each supertype on LRS and the population frequency of supertypes  
354 (effect = -0.004, SE = 0.002, p-value = 0.023; Figure 5a). We found a similar pattern when  
355 evaluating this relationship for the survival estimates, but it was not statistically significant  
356 (effect = -0.006, SE = 0.005, p-value = 0.279; Figure 5b).

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358

359

## 360 **Discussion**

361 This study thoroughly investigated the effects of MHC class II variation on two key  
362 components of individual fitness, survival and lifetime reproductive fitness, in a wild  
363 migratory passerine. Individuals with supertype 12 and supertype 11 had lower lifetime  
364 number of fledglings while those with supertype 7 were favoured in LRS. In addition,  
365 individuals with supertype 12 had lower survival rates, suggesting that a lower survival  
366 mediated the lower reproductive success related to MHC composition. Furthermore, we found  
367 a negative relationship between the effect of supertypes on fitness and their population  
368 frequency, with the most common being detrimental (supertype 11 = 88.89 %; supertype 12 =  
369 73.74 %) and the rarest (supertype 7 = 3.03 %) beneficial to the fitness of individuals.  
370 Overall, these findings suggest that the current frequency of supertypes in the population  
371 results from a coevolutionary arms race with pathogens, with the frequency of a supertype  
372 decreasing as the pathogens evolve resistance to it.

373 Several works show that MHC diversity affects individual fitness (Møller et al. 1990,  
374 Borghans et al. 2004, Kutzer and Armitage 2016) where individuals with high (Doherty and  
375 Zinkernagel 1975, Thoß et al. 2011, Radwan et al. 2012) or intermediate (Wegner et al.  
376 2003a, Bonneaud et al. 2004, Madsen and Ujvari 2006, Kalbe et al. 2009) levels of MHC  
377 diversity often enjoy higher fitness. In contrast, we found that overall diversity, either in its  
378 linear (*maximal diversity hypothesis*) or quadratic term (*optimal diversity hypothesis*), do not  
379 affect survival or the LRS of individuals. It seems, therefore, that overall MHC diversity has  
380 no significant consequences for the fitness of pied flycatchers. Thus, the identity of the  
381 supertypes possessed by an individual may underlie the MHC-mediated effects on fitness  
382 (Sepil et al. 2012, Grogan 2014, Halvarsson 2016, Pierini and Lenz 2018). According to this  
383 idea, individuals carrying the rarest supertype (supertype 7) had higher lifetime reproductive  
384 success, whereas two of the most common supertypes in the population (supertypes 11 and

385 12) conferred lower survival and, consequently, lifetime reproductive success. Further, the  
386 effect of each supertype on fitness were negatively related to their frequency in the  
387 population. Taking together, these results suggest that the frequency of those superotypes has  
388 been increasing until they have gone from advantageous to detrimental, because the pathogen  
389 has adapted to them (Ejsmond and Radwan 2015).

390         Given the observational nature of this study, we can only speculate on the exact  
391 mechanisms underlying the superotypes-fitness relationships. Differences in fitness related to  
392 MHC variation may depend on conditions experienced in early life, including environmental  
393 conditions (Reid et al. 2003, Öberg et al. 2015), parental effort (Alatalo et al. 1988), but also  
394 exposure to pathogens that would be crucial for future fitness (Evans et al. 2016). Further, if  
395 breeding adults are subjected to chronic infections, they may have smaller egg clutches  
396 because they are immunosuppressed and thereby lower reproductive success (Richner et al.  
397 1995, Oppliger et al. 1996, Siikamäki et al. 1997). Individuals carrying certain superotypes  
398 may also show low survival prospects because they cannot withstand the physiological stress  
399 and energetic demands required for certain life stages, such as migration (Altizer et al. 2011),  
400 or because their ability to escape from potential predators is impaired (Garamszegi et al.  
401 2015).

402         We found that some superotypes affected only lifetime reproductive success (supertype  
403 7 and supertype 11) while another supertype also affected survival (supertype 12).  
404 Discrepancies in the effect of certain superotypes on different fitness components might arise  
405 due to several reasons. First, as the highest mortality from pathogen infection is usually  
406 reported when individuals are first exposed (Atkinson and Samuel 2010), it is possible that  
407 detectable effects of certain superotypes on survival occurred at earlier life phases, such as the  
408 nestling or fledgling phases, not considered in the present study. Second, a given supertype  
409 might confer resistance to pathogens that affects only one component of fitness or affect it to

410 a greater extent than others components. For example, certain pathogens may induce  
411 reproductive costs at a single or multiple breeding stages, such as hatching success,  
412 provisioning rates and fledgling success (Knowles et al. 2010, Sepil et al. 2012), thus  
413 affecting overall reproductive success. Alternatively, other pathogen may affect survival more  
414 than reproduction if, for example, these are transmitted mostly around migration periods, i.e.  
415 late summer and early spring rather than during the breeding season (Cosgrove et al. 2008).  
416 Also, some pathogen might affect the physical condition of infected individuals during  
417 migration, thereby affecting their survival (Risely et al. 2017). In this line, in a population of  
418 Bewick's swans, it was found that those individuals infected by Influenza A virus could store  
419 less food, and thus their preparation for migration was negatively affected (Van Gils et al.  
420 2007).

421         Although MHC fitness associations reported here overall support the *rare-supertype*  
422 *advantage hypothesis* as the main mechanism driving MHC variation, we cannot completely  
423 rule out that the *fluctuating selection* is also operating in our population. Changing  
424 environmental conditions may influence MHC variation by altering the prevalence of  
425 different pathogens over time and space (Awadi et al. 2018), which, in turn, should exert  
426 positive selection on supertypes conferring protection against these new common pathogens.  
427 Since such selection process is under the pressure of environmental factors, rather than  
428 coevolutionary arms race between hosts and pathogen, a strong relationship between fitness  
429 and the frequency of each supertype in the population is not expected. However, although  
430 unlikely, such negative relationship between frequency and fitness might arise under very  
431 particular circumstances, as for example, whether the number of supertypes in the species is  
432 low, the selected supertypes were previously rare in the population and benefit provided by  
433 rare supertypes is very high relative to common. Therefore, to complete rule out and/or  
434 disentangle the relative effect of both mechanisms (*fluctuating selection* and *rare-supertype*

435 *advantage*) it would be necessary to investigate (i) MHC-fitness association over long period  
436 of time and/or in different sites (Hacking et al. 2018) or (ii) whether MHC variation is driven  
437 by specific environmental factors and/or pathogens. As this is extremely challenging in  
438 nature, few studies have been able to find *fluctuating selection hypothesis* acting in a single  
439 population (Hedrick 2002, Westerdahl et al. 2004).

440 In conclusion, our findings show the importance of MHC specific supertypes (11, 12  
441 and 7), but no effect of MHC diversity, in individual fitness by mediating their lifetime  
442 reproductive success and survival. Further, these fitness effects seem to be mainly driven by  
443 the *rare supertype advantage* mechanism, as the frequency of supertypes in the population  
444 was negatively related to the advantage conferred on individual fitness. However, interesting  
445 avenues remain to be explored. For example, identify the pathogen(s) underlying the between-  
446 individual differences in fitness related to supertypes 11, 12 and 7. In addition, it would be of  
447 particular interest to disentangle the relative influence, if any, of *fluctuating selection* in  
448 relation to the *rare-supertype advantage* mechanisms on the maintenance of MHC class II  
449 diversity of pied flycatchers.

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730 **Tables and figures**

731 Table 1. Generalized linear models (GLMM) used to estimate the effect of the MHC diversity  
 732 on the lifetime fledglings of pied flycatchers.

Model	Predictor	np	$\beta$	SE	p-value	AICc	$\Delta$ AICc
<b>1</b>	<b>Supertype 12</b>	<b>6</b>	<b>-0.346</b>	<b>0.1</b>	<b>0.007</b>	<b>591.95</b>	<b>-9.27</b>
<b>2</b>	<b>Supertype 11</b>	<b>6</b>	<b>-0.435</b>	<b>0.13</b>	<b>0.0013</b>	<b>593.17</b>	<b>-8.05</b>
<b>3</b>	<b>Supertype 7</b>	<b>6</b>	<b>0.632</b>	<b>0.23</b>	<b>0.0107</b>	<b>596.98</b>	<b>-4.24</b>
4	nStypes	6	-0.071	0.04	0.0455	599.49	-1.73
5	Supertype 8	6	-0.159	0.10	0.1072	600.89	-0.33
Null	Null	5	NA	NA	NA	601.22	0.00
6	nStypes <sup>2</sup>	7	-0.913	0.45	0.1353	601.80	0.58
7	Supertype 1	6	-0.133	0.12	0.2826	602.33	1.11
8	Supertype 6	6	-0.128	0.17	0.4439	602.90	1.68
9	Supertype 14	6	0.076	0.10	0.4594	602.94	1.72
10	Supertype 9	6	0.063	0.10	0.5237	603.08	1.86
11	Supertype 3	6	-0.105	0.29	0.7121	603.35	2.13
12	Supertype 13	6	0.028	0.10	0.7703	603.40	2.18
13	Supertype 5	6	0.023	0.11	0.8329	603.44	2.22
14	Supertype 10	6	-0.027	0.14	0.8496	603.45	2.23
15	Supertype 2	6	0.013	0.110	0.8925	603.47	2.25
16	Supertype 4	6	0.005	0.12	0.9630	603.49	2.27

733 Selection models was based on Akaike's Information Criterion corrected for small sample  
 734 sizes (AICc);  $\Delta$ AICc, difference in AICc between the null model and the AICc of each model;  
 735 nStypes and nStypes<sup>2</sup>, linear and quadratic covariate of the number of supertypes,  
 736 respectively; np, number of estimable parameters;  $\beta$ , effect of the focal predictor; SE, standard

737 error; p-value, p-value calculated with likelihood ratio test respect to the null model and with  
738 Benjamini-Yekutieli correction; Significant variables in bold.

739 Table 2. Results of capture-recapture analyses modelling the effect of MHC diversity on  
 740 apparent survival probabilities ( $\Phi$ ) of pied flycatchers.

No	Model	k	Deviance	AICc	$\Delta$ AICc
<b>1</b>	<b>Base + ST12</b>	<b>11</b>	<b>284.651</b>	<b>307.998</b>	<b>-6.946</b>
2	Base + ST11	11	291.106	314.453	-0.491
3	Base + ST7	11	291.551	314.898	-0.046
Base	Base	10	293.828	314.944	0
4	Base + ST14	11	291.799	315.146	0.202
5	Base + ST3	11	292.820	316.167	1.223
6	Base + ST13	11	292.958	316.305	1.361
7	Base + ST6	11	293.530	316.877	1.933
8	Base + ST9	11	293.577	316.924	1.98
9	Base + ST10	11	293.709	317.056	2.112
10	Base + nStypes	11	293.757	317.104	2.16
11	Base + ST5	11	293.764	317.110	2.166
12	Base + ST4	11	293.799	317.146	2.202
13	Base + ST2	11	293.803	317.150	2.206
14	Base + ST8	11	293.803	317.150	2.206
15	Base + ST1	11	293.827	317.174	2.23
16	Base + nStypes <sup>2</sup>	12	293.742	319.342	4.398

741 k, number of parameters; AICc, Akaike's Information Criterion corrected for small sample  
 742 sizes;  $\Delta$ AICc, difference in AICc between the base model and the AICc of each model; ST,  
 743 supertype; nStypes and nStypes2, linear and quadratic covariate of the standardized number  
 744 of superotypes, respectively; "+", indicates additive effects. The base model corresponds to " $\Phi$   
 745 (birthyear + agereprod1) p (sex)", where the probability of survival ( $\Phi$ ) depends on the

746 birthyear (birthyear) and the age at which the first reproduction is recorded (agereprod1= one-  
747 year-old or >1yo). Resighting probability ( $p$ ) was modelled as sex-dependent. Model shown  
748 in bold is the most parsimonious model supported by the data.

749 Figure 1. Frequencies of each supertype present in the pied flycatcher sample (n= 99) used for  
750 survival and reproductive success analyses. Supertypes 11 and 12 are shown in red and  
751 supertype 7 in blue.

752

753 Figure 2. Effect of specific MHC class II supertypes, (a) supertype 12, (b) supertype 11 and  
754 (c) supertype 7, on lifetime fledglings of pied flycatchers (partial effect with 95% CI).

755

756 Figure 3. Estimates of survival rates ( $\pm$  SE; averaged over age at first reproduction) in pied  
757 flycatchers according to their birth year. Solid bars indicate individuals that carry supertype  
758 12 while dotted bars indicate individuals lacking supertype 12.

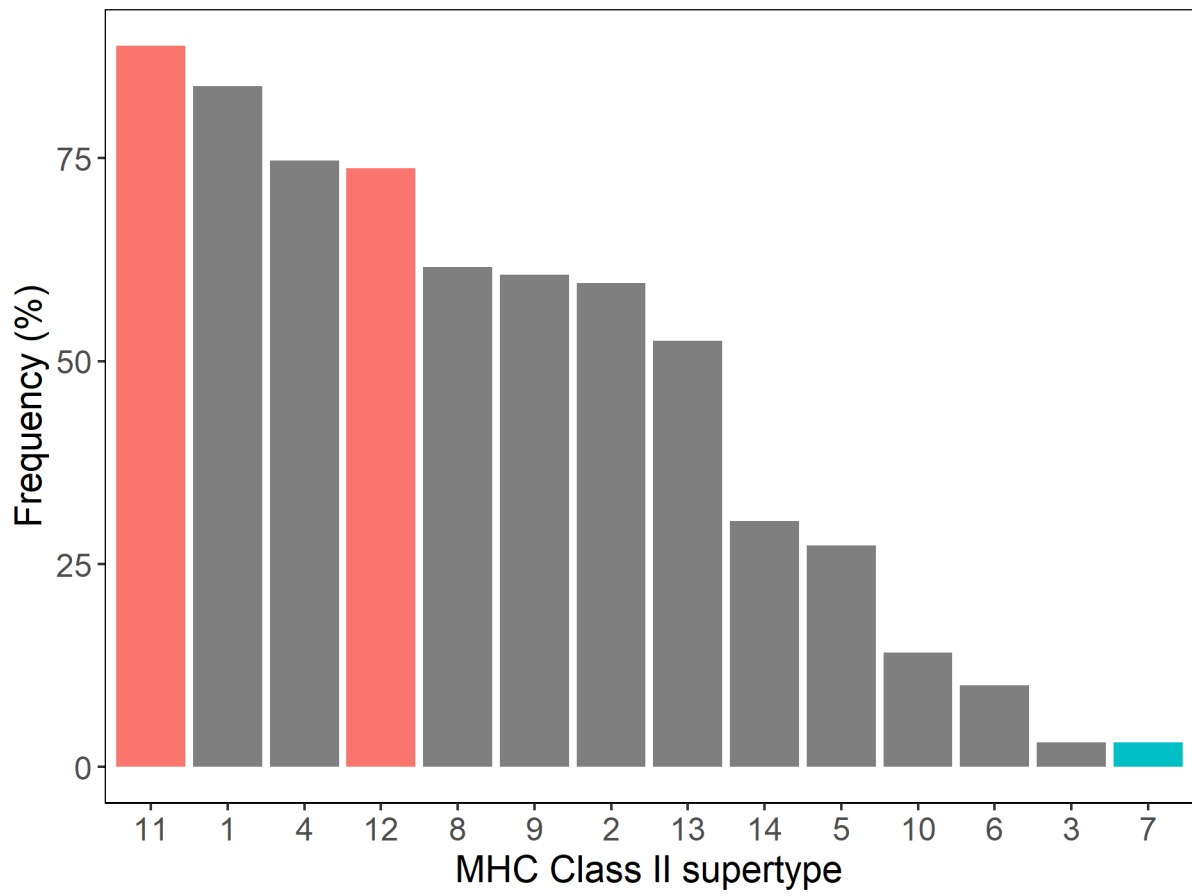
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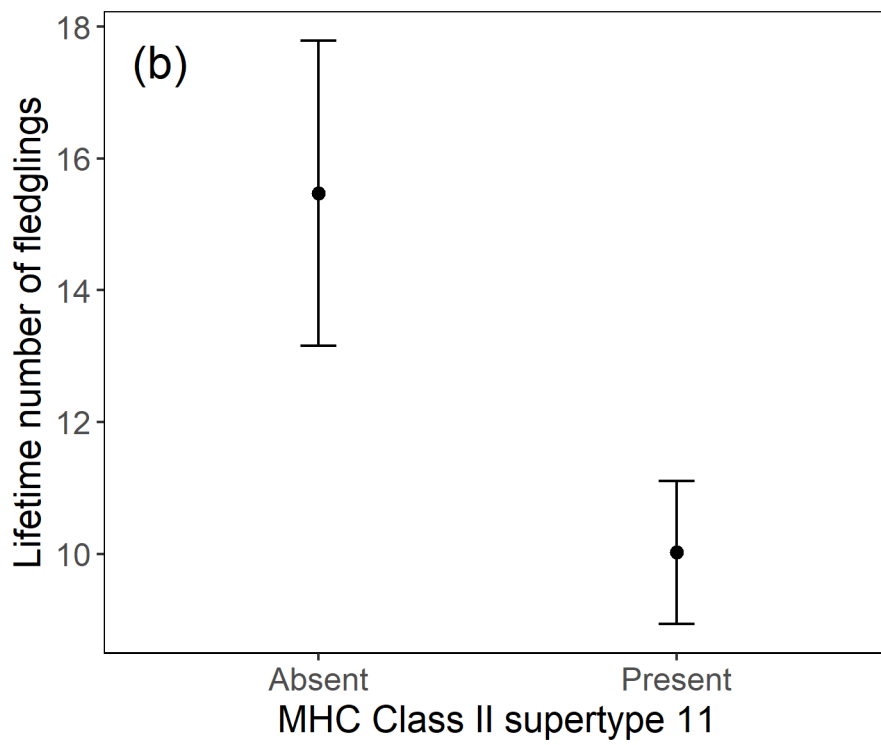
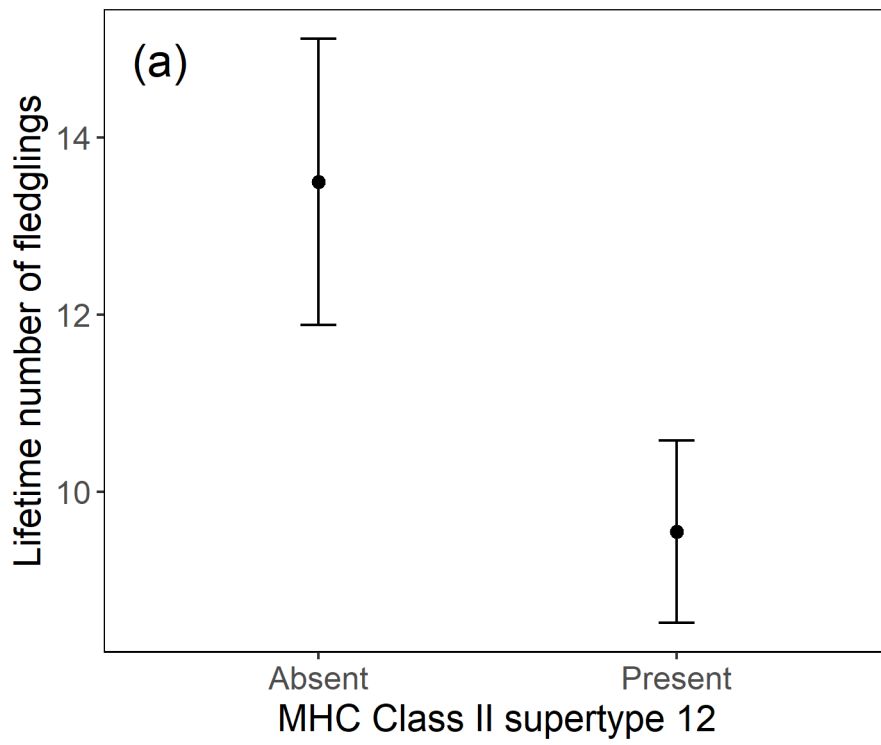
760 Figure 4. Effect size estimates (beta coefficients with 95% CI) of the variables of interest, i.e.,  
761 those covariates with a lower AICc than the base model in Table 1. Values below zero  
762 indicate negative associations between MHC and individual survival.

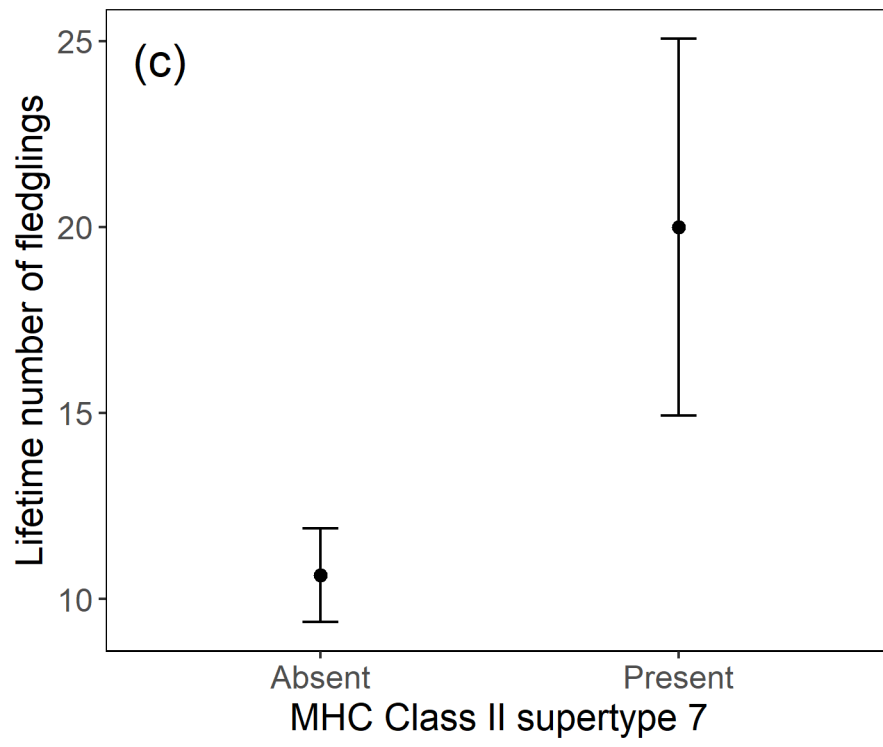
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764 Figure 5. Linear regression between population frequency of each supertype and their  
765 estimated effects on (a) lifetime reproductive success (LRS) and (b) apparent survival.

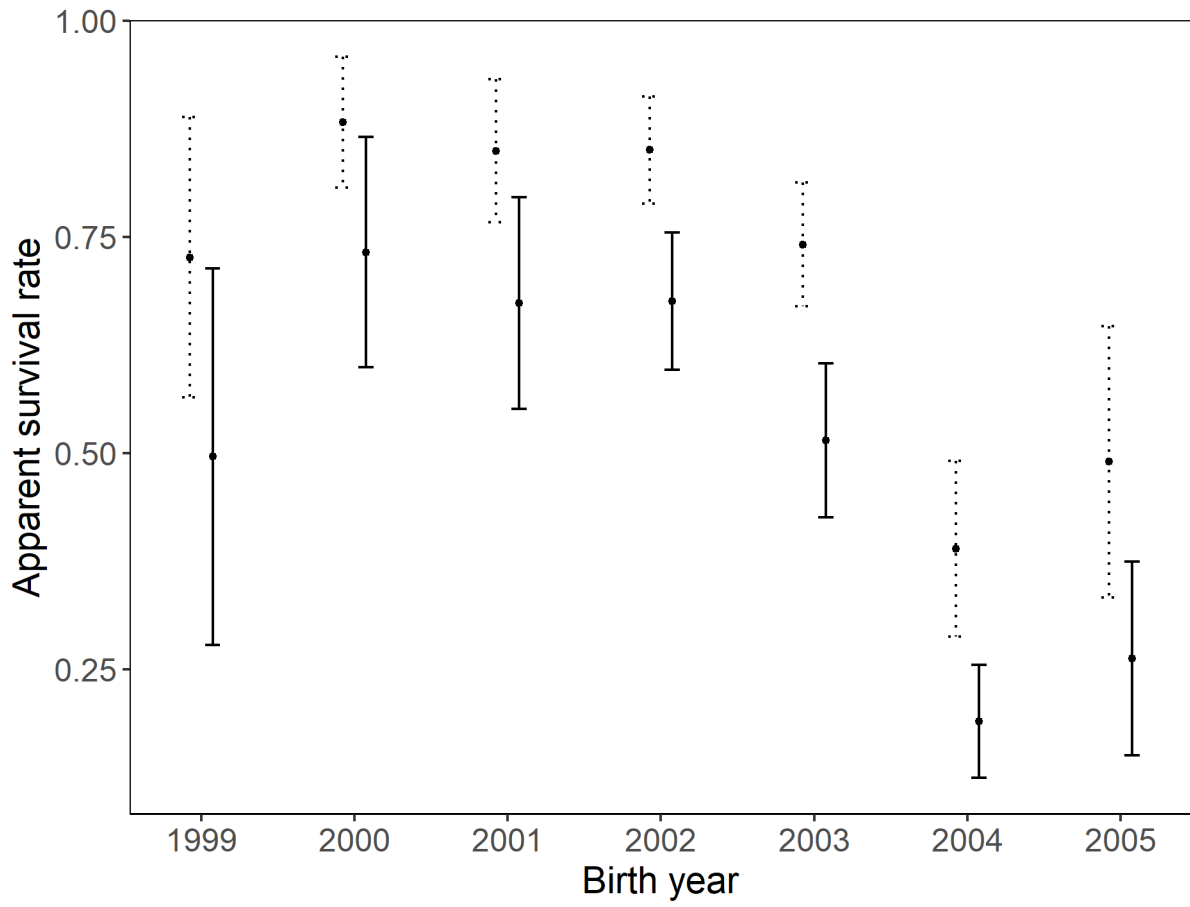
766 Figure 1.







768 Figure 3.



769 Figure 4.

