

1 **Evidence of non-random mating in a colour polymorphic raptor, the**
2 **Booted eagle**

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28 **Abstract**

29 Sexual selection and non-random mating are considered, among others, determinant
30 mechanisms for the maintenance of genetic colour polymorphism in some bird species.
31 We analyse the mechanisms, which, in parallel with Mendelian inheritance, may be
32 acting in the maintenance and evolution of the morph ratio in a two-morph raptor
33 species, using observational data of successful breeding individuals and their offspring

34 from long-term studies conducted in three Spanish populations. Our results showed that
35 the dark offspring produced in breeding events involving mixed-morph adult pairs far
36 exceeds the expected value under the Hardy-Weinberg equilibrium, especially in the
37 case of pairs formed by a light male and a dark female. In addition, the low number of
38 dark eaglets born from pairs formed by light individuals (indistinctly homozygous or
39 heterozygous) indicates that the number of breeding events of heterozygous (both the
40 male and female) light morph pairs, was much lower than expected. As the plausible
41 existence of a transmission ratio distortion phenomenon in heterozygous light morph
42 males does not, alone, explain the disproportionate number of dark eaglets observed,
43 our results suggest that one or two selective mating phenomena may be occurring in this
44 polymorphic system. The first one could be a disassortative mating process whereby
45 heterozygous light males preferentially mate with dark females, based on the imprint of
46 the colour morph of their mother. The second phenomenon would only affect light
47 morph individuals, which would preferentially mate with heterozygous individuals of
48 the opposite sex, selected according to secondary sexual characteristics or behavioural
49 traits that are unknown at the moment.

50 **Keywords:** colour polymorphism, disassortative mating, mate choice, Mendelian
51 inheritance

52

53 **Introduction**

54 The study of factors involved in the maintenance of genetic colour polymorphism in
55 animal species has been subject of intensive research for decades (Sinervo and Lively
56 1996; Roulin 2004; Gray and McKinnon 2007; Wellenreuther et al. 2014). In the case
57 of bird species, three main mechanisms have been hypothesized to explain the
58 maintenance of colour polymorphism: apostatic, disruptive and sexual selection
59 (Galeotti et al. 2003). While the apostatic and disruptive selection hypotheses postulate
60 differential foraging efficiencies for the morphs, either in the form of an “avoidance
61 image” (Rohwer and Paulson 1987) or based on the advantages of foraging in different
62 habitats and/or light conditions and preying upon different species (Roulin and Wink
63 2004; Tate and Amar 2017), respectively, the sexual selection hypothesis proposes that
64 the maintenance of different colour morphs may depend on preferences at the time of
65 mating (Galeotti et al. 2003). In addition, there are other mechanisms such as
66 antioxidant levels (Galván et al. 2010) and parasitic loads (Lei et al. 2013), that may
67 affect each morph differently and so condition the colour polymorphism balance.

68 Although sexual selection and mate choice are two important processes in colour
69 polymorphic systems, maintaining colour polymorphism also requires some form of
70 correlational (with other traits) or balancing selection (e.g. heterozygote advantage or
71 negative frequency-dependent selection) underlining the complexity of the mechanisms
72 involved (Wellenreuther et al. 2014).

73 Mate choice is an important evolutionary process and a very active area of
74 research in population genetics, animal behaviour and evolutionary ecology (Edward
75 2015; Carvajal-Rodríguez 2018). This may be assortative, when individuals prefer to
76 mate with similar partners, or disassortative, when the preference is for opposite morphs
77 (Roulin and Bize 2007). Disassortative mating favours outbreeding or the production of

78 heterozygous offspring (Galeotti et al. 2003; Roulin and Bize 2007) and can maintain
79 genetic diversity through negative frequency-dependent morph mating (Wellenreuther
80 et al. 2014).

81 A number of factors determining mate choice have been described in bird
82 species, including, body size (Forero et al.2001; Quinard et al. 2014; Indykiewicz et al.
83 2017); telomere length and intrinsic quality (Khoriauili et al.2017; Schull et al.2018),
84 habitat quality (Ferrer and Penteriani 2003), ornaments in the plumage (MacDougall
85 and Montgomerie 2003), plumage colour traits (Bortolotti et al. 2007; Pryke and
86 Griffith 2007; Rowe and Weatherhead 2011; Semenov et al.2017), plumage differences
87 (Warkentin et al. 1992), phenotypic similarity (Bolnick and Kirkpatrick 2012; Class and
88 Brommer 2018), different phenotypes (Hedrick et. al.2018), calls (Snowberg and
89 Benkman 2007), heterozygosity (García-Navas et al.2009; Ortego et al. 2009; Zandberg
90 et al.2017), and social and behavioural features, such as familiarity (Senar et al. 2013),
91 learning mate preferences (Gilman and Kozak 2015) and imprinting on parents (Cooke
92 and McNally 1975; ten Cate and Vos 1999; Grant and Grant 2018, Krüger et al. 2001).

93 The booted eagle is a medium-sized, forest-dwelling migratory raptor, which arrives in
94 its Palaearctic breeding quarters from late March to early May (Cramp and Simmons
95 1980; Ferguson-Lees and Christie 2001). Females lay one or two eggs (sometimes
96 three) and normally raise one or two (rarely three) young in each breeding season
97 (Ferguson-Lees and Christie 2001). The booted eagle has two distinct morphs, light
98 (pale) and dark, although several authors recognize an intermediate or ‘rufous’ morph
99 for some individuals of the more variable dark morph (Clark 1999; García-Dios 2017).
100 Bosch et al. (2019) suggested that the inheritance of this polymorphism follows the
101 Mendelian inheritance pattern, with an alternative system comprising two epistatic loci
102 and two alleles per locus, which could explain the occurrence of the more variable dark

103 morph plumages. Therefore, light morph eagles can be homozygous (LL) or
104 heterozygous (Ld), while the dark morph eagles are always homozygous (dd).

105 In this communication we analyse the mechanisms that, apart from Mendel's
106 first law, may be acting in the maintenance and evolution of the morph ratio in this
107 species, using observational data on plumage colouration of successful breeding pairs
108 and eaglets from long-term studies conducted in three Spanish populations of this
109 species.

110 Our study mainly focuses on mixed colour pairs and also on the reproductive
111 heterozygous pairs of the light morph, which seem to be fewer than might be expected
112 according to the Hardy-Weinberg equilibrium. The study also aims to estimate the
113 expected phenotypic and genotypic frequencies in the offspring, assess possible
114 deviations from these frequencies (assuming random mating and Hardy-Weinberg
115 equilibrium conditions) and discuss plausible explanations for the observed morph
116 frequencies based on genetic processes and/or existing hypotheses about mate-choice
117 behaviour.

118

119 **Methods**

120 We studied three Spanish booted eagle populations located in central Catalonia, Els
121 Ports Natural Park (South Catalonia) and the Special Protection Area “Sierras de
122 Burete, Lavia y Cambrón”, in the centre of Murcia province (Fig. 1), characterized
123 mainly by both Eurosiberian and Mediterranean forests of Black Pine *Pinus nigra* and
124 Aleppo Pine *Pinus halepensis*, respectively, interspersed with scrubland and cultivated
125 areas. A detailed description of the study areas can be found in Bosch et al. (2015,
126 2019).

127 Information about the colour morph of successful breeding adult individuals and
128 their offspring was obtained between 1995 and 2018, during the field work carried out
129 to study the breeding biology of the species (Bosch 2003; Martínez et al. 2006a; 2006b;
130 Pagán et al. 2009; Bosch 2011; Baiges 2014; Jiménez-Franco et al. 2013; Bosch et al.
131 2015). In total, 44 territories were monitored in central Catalonia, 17 in Els Ports
132 Natural Park and 57 in the province of Murcia. From the end of March to the beginning
133 of May the occupation of the territories was determined by direct observation of
134 territorial flights, mating calls or the existence of at least one nest repaired with new
135 material or the observation of the female incubating eggs (Jiménez-Franco et al.
136 2011). The sex of each adult eagle was easily determined by its size and by its
137 reproductive behaviour (Jiménez-Franco et al. 2013).

138 In this study the data of the three populations are pooled for two reasons: 1) to increase
139 the sample size and therefore the reliability of the results and 2) the allele frequencies
140 for the three populations are very similar (e.g. the proportion of dark morphs varies
141 from 8.44% in Murcia to 9.3% in Els Ports Natural Park). Data concerning the colour
142 morph of the successful breeding pairs and their offspring during the study period were
143 used, although no data on the sex of fledglings were collected.

144 In breeding events of mixed pairs comprising a light morph male and a dark morph
145 female, we knew that the male was heterozygous if mating produced dark chicks. On
146 the other hand, if only light morph chicks were produced, the male could have been
147 homozygous or heterozygous. In the inverse case of mixed pairs comprising a light
148 morph female and dark morph male, same criteria were followed, but in this case, for
149 the female. In the case of reproductive events where both members were of the light
150 morph, we only knew that both adults were heterozygous if they raised dark chicks. In

151 all other cases, there were two other possibilities, that both were homozygous or that
152 only one of them was homozygous.

153 The allelic frequencies of successful breeding adults were calculated using the Hardy-
154 Weinberg equilibrium equation $p^2 + 2pq + q^2 = 1$ (in our case $L^2 + 2Ld + d^2 = 1$) to estimate
155 the frequency of the carrier state ($2pq$) for an autosomal recessive trait from the
156 proportion recessive dark morph individuals. Allelic frequencies between males and
157 females were differentiated because the proportion of dark morph females almost
158 doubled that of dark morph males. Thus, taking into account random mating, it was
159 possible to calculate the probability of the expected breeding pairs for each of the nine
160 possible combinations. For example, for a breeding pair consisting of a male of the
161 heterozygous light morph and a female of the homozygous dark morph, the probability
162 would be 0.3823 (probability σLd) \times 0.1242 (probability φdd) = 0.04748 (probability
163 $\sigma Ld \times \varphi dd$); $0.04748 \times 100 = 4.748\%$, where 50% of the offspring would be
164 heterozygous of the light morph (Ld) (2.374%) and the other 50% homozygous of the
165 dark morph (dd) (2.374%). By contrast, for a breeding pair consisting of a male of the
166 homozygous light morph and a female of the homozygous dark morph the probability
167 would be 0.5515 (probability σLL) \times 0.1242 (probability φdd) = 0.0685 (probability
168 $\sigma LL \times \varphi dd$); $0.0685 \times 100 = 6.85\%$, where 100% of the offspring would be
169 heterozygous of the light morph (Ld). Thus, under the ideal conditions of the Hardy-
170 Weinberg equilibrium, there would be a total of 9.224% ($2.374\% + 6.85\% = 9.224\%$)
171 heterozygous chicks of the light morph and 2.374% homozygous chicks of the dark
172 morph, which, by simplifying, gives an expected ratio of 3.89: 1 (light / dark). As the
173 studied eagles were not tagged and individuals can change from one year to the next, we
174 treated each reproductive event as if it involved different individuals. We are aware that
175 this procedure could give rise to biases by pseudo-replication, since the same

176 individuals / pairs might be repeated for several years. However, in a study carried out
177 on the population of Murcia, the average period of occupation of a territory by an
178 individual was only 1.89 ± 1.25 (SD) years, being 2.06 ± 1.39 (SD) years ($n = 17$) for
179 females and 1.60 ± 0.97 (SD) years ($n = 10$) for males, regardless of the colour morph
180 (Jiménez-Franco et al. 2013), so the impact of the biases produced by pseudo-
181 replication would be very low and affect all couples with different combinations of
182 phenotype and / or genotype in the same way. No fitness differences were detected
183 between the different combinations of phenotypes and / or genotypes that could explain
184 deviations in the Hardy-Weinberg equilibrium, since no significant differences were
185 found in the reproductive success (Martínez et al. 2016).

186

187 **Results**

188 Between 1995 and 2018 a total of 483 successful breeding events were monitored in the
189 three populations studied, in which 32 of the adult males involved and 60 of the adult
190 females were of dark morph. From these numbers, assuming the Hardy-Weinberg
191 equilibrium, the overall estimated allele frequencies would be $L = 0.743$ and $d = 0.257$
192 for males, and $L = 0.648$ and $d = 0.352$ for females, which implies the following
193 expected genotype frequencies in the population: 55.15% (LL), 38.23% (Ld) and 6.63%
194 (dd) for males, and 41.93% (LL), 45.65% (Ld) and 12.42% (dd) for females.

195 As a result of the 483 breeding events, a total of 677/76 light/dark eaglets flew
196 (Table 1). The 58 (12%) breeding events involving light♂/dark♀ mixed-morph adult
197 pairs produced 53 dark eaglets and another 30 (6.21%) breeding events of dark♂/light♀
198 mixed-morph pairs produced 15 dark eaglets. This number far exceeds the expected
199 value under Hardy-Weinberg equilibrium conditions, especially in the case of pairs
200 formed by a light male and a dark female (Fig. 2), for which the observed ratio of

201 light/dark eaglets (0.72:1) was significantly lower than expected (3.89:1) (Table 1; $\chi^2 =$
202 25.024, $P < 0.001$). In the case of dark male-light female pairs, the observed ratio of
203 light/dark eaglets produced (1.93:1) was also lower than expected (2.84:1) (Fig.2; Table
204 1), although the difference was not statistically significant ($\chi^2 = 0.491$, $P=0.483$).
205 Furthermore, only five dark eaglets were born from pairs formed of light individuals
206 (indistinctly homozygous and heterozygous), which means that, theoretically, there
207 were only about 20 breeding events of heterozygous (both the male and the female)
208 light morph pairs, which is much less than the 84 expected (Table 1). The observed
209 light/dark eaglet ratio (122:1) for light-light pairs was significantly higher than expected
210 (17.75:1) (Table 1; $\chi^2 = 19.796$, $P < 0.001$).

211 Overall, assuming the Hardy-Weinberg equilibrium, the allelic frequencies for
212 fledglings were $L = 0.682$ and $d = 0.318$, which means an increase of more than 1.8%
213 over the expected frequency of the recessive allele ($d = 0.300$).

214 **Discussion**

215 Our results show that the number of dark eaglets produced by mixed-morph breeding
216 pairs far exceeded the number expected under the Hardy-Weinberg condition. The
217 accuracy of the Hardy-Weinberg equilibrium equation relies on several assumptions,
218 such as large population, no gene flow, no selection, random allele transmission and
219 random mating, the last three unlikely to be strictly true for the populations studied. The
220 observed significant departure from the expected Mendelian transmission of alleles
221 could be attributed to a transmission ratio distortion (TRD) effect (Huang et al. 2013),
222 which occurs when one of the two alleles from either parent is preferentially transmitted
223 to the offspring. In fact, Bosch et al. (2019) suggested the occurrence of TRD in booted
224 eagle light morph heterozygous males in our populations. In males, cases where sperm
225 with a certain genotype have been able to interrupt their competitors with a different

226 genotype have been described, as in the case of the house mouse and *t*-alleles (Lewontin
227 & Dunn 1960, Lewontin 1968, Lyon 1984) and a segregation distorter in *Drosophila*
228 *melanogaster* (Hiraizumi *et al.* 1960, Hartl *et al.* 1967). However, even if all the light
229 morph heterozygous males that paired with dark morph females had transmitted the
230 recessive allele in all cases (i.e. producing only dark morph eaglets), a light/dark eaglet
231 ratio of 1.44:1 would be expected, which is still far from the 0.72:1 ratio observed
232 (Table 1).

233 Therefore, in addition to the TRD effect in booted eagle light heterozygous
234 males, another process or processes might exist to explain the differences observed. At
235 the time of leaving the nest, when the colour morph data were recorded, the offspring
236 have not yet suffered any other selective pressure that could alter the proportion of
237 morphs, other than to which they may have been exposed in the nest. In this sense,
238 Galván *et al.* (2010) found that the chicks of the dark morph had significantly lower
239 levels of glutathione (tGSH) than those of light morph and also that female chicks of the
240 dark morph were significantly in better physical condition than light morph ones, which
241 was not the case with males. Moreover, several research works on different animal taxa,
242 including raptors, found a relationship between different colour morphs and the
243 endoparasite incidence, with dark morphs having lower parasitic loads (Chakarov *et al.*
244 2008, Jacquin *et al.* 2011, Lei *et al.* 2013, MacColl *et al.* 2013). Thus, dark morph
245 individuals have a selective advantage and may become more abundant than those of
246 the light morph. However, related to this, although the productivity of breeding events
247 involving dark morph individuals productivity was found to be slightly higher than that
248 of events only involving light morph individuals, the difference was not statistically
249 significant (Martínez *et al.* 2016), so any impact of the above phenomena on the morph
250 ratio would be very low. Our results suggest a disassortative mating phenomenon in

251 which heterozygous light males preferentially mate with dark females. However, even if
252 all light morph males that mated with dark females were heterozygous, the expected
253 morph ratio in the offspring would be 1: 1 light / dark, still far from the 0.72: 1
254 light/dark obtained, so this result can be explained with the additive concurrence of
255 TRD, although the degree of influence of each factor is impossible to determine.
256 Among the causes underlying the process of selective mating, one plausible explanation
257 could be the imprint of the chicks on the parental phenotypes, determining their future
258 preferences at the time of mating (Cooke and McNally 1975; ten Cate and Vos 1999;
259 Krüger et al. 2001). For example, Grant and Grant (2018) provided statistical evidence
260 that two species of Darwin's finches imprint on morphological cues of their parents and
261 mate assortatively. The evidence of imprinting was stronger in the case of sons than
262 daughters and also stronger for imprinting on fathers than on mothers. As is the case
263 with Common Buzzard *Buteo buteo* (Krüger et al. 2001), in the booted eagle, the
264 females spend most of their time brooding, feeding and watching the chicks until they
265 reach approximately 30 days of age (García-Dios 2017). For they part, in that period,
266 males only approach the nest to deliver captured prey to the female, usually near the
267 nest, although they may deliver it to the nest itself, but leave quickly and not participate
268 in the feeding tasks. Thus, the light heterozygous eaglets would be preferentially
269 imprinted on the colour of their mother and this phenomenon could influence their
270 preferences when it comes to mating, especially in the case of males. In this sense, 80%
271 of the breeding events involving a light morph male and dark morph female in our
272 studied populations produced dark chicks, demonstrating the male's colour
273 heterozygosity. In the reverse case, of mixed pairs involving a light morph females, the
274 phenomenon of the TRD would not occur since the dark males are always homozygous.
275 However, although also it may have happened the selective mating of light morph

276 heterozygous females with dark morph males, or a non-random transmission (TRD) of
277 the allele by females, these factors would not be very intense since, despite the fact that
278 the proportion of dark chicks was somewhat higher than expected, the difference was
279 not statistically significant. For this combination of morphs only about 45% of the
280 breeding events produced dark morph chicks.

281 However, the phenomenon of selective mating caused by imprinting does not, alone,
282 explain the low number of breeding events involving light morph heterozygous pairs,
283 since the number of dark morph individuals, both male and female, in the studied
284 populations was very low and the number of expected light morph heterozygous
285 individuals, both male and female, was very high. This fact also suggests the existence
286 of selective mating based on heterozygosity, in which the light morph homozygous
287 individuals would preferentially mate with heterozygous individuals of their same
288 colour morph or vice versa, hindering the formation of heterozygous light morph pairs,
289 and explaining the low number of light morph heterozygous pairs found in our results.

290 The phenomenon of heterozygosity-based assortative or disassortative mating
291 has been studied in several bird species, such as the blue tit *Cyanistes caeruleus*
292 (García-Navas et al. 2009) and the lesser kestrel *Falco naumanni* (Ortego et al. 2009),
293 using micro-satellite genetic markers. In these cases, it was demonstrated that in
294 reproductive events in which heterozygous individuals are involved, the clutch size and
295 fledgling success are higher, as is the quality of the offspring produced. By contrast, and
296 as with the common buzzard *Buteo buteo* (Boerner et al. 2013), this correlation of
297 heterozygosity and fitness does not seem to occur in our case. Although productivity
298 was higher in breeding events involving dark morph individuals than in events
299 involving only light morph individuals, the difference was not statistically significant
300 (Martínez et al. 2016) so the level of colour heterozygosity, in our case it also does not

301 seem to be determinant of reproductive success. Neither do we know the
302 heterozygosity-wide level of the light morph individuals colour heterozygous.

303 Contrary to other species in which the mating preferences of both sexes are
304 known (Pryke and Griffith 2007), in the booted eagle the mechanisms involved in the
305 choice of mate and in the role played by each of the pair members are completely
306 unknown, as are the secondary sexual characteristics related to the genetic variability
307 that can be identified by the other individuals. The quantity, size and intensity of the
308 dark spots on breast could be a differentiating element among light morph individuals,
309 but their relationship with heterozygosity should be checked with genetic markers.

310 Another possible scenario could be one in which the homozygous females of both
311 colour morphs preferentially select the light morph heterozygous males. Female mate
312 choice has typically been considered a strong selective force for maintaining selective
313 mating in polymorphic species (Fox et al. 2002; Pryke and Griffith 2007).

314 Mate choice can have important consequences because it influences selective
315 mating and the level of genetic variation maintained within populations. In species with
316 genetically determined polymorphism, such as booted eagle, non-random mate choice
317 may affect the evolution, stability and maintenance of the alternative phenotypes.
318 Dissortative mating clearly prevents the loss of the recessive or rare phenotypes
319 (Knapton and Falls 1983; Pryke and Griffith 2007). In our case, the apparent immediate
320 consequence of the non-random transmission of the dark allele and/or of selective
321 mating was an increase of approximately 1.8% in the presence of the dark allele in the
322 eaglets produced with respect to the expected frequency. This fact may be important for
323 the maintenance of the dark colour morph in populations where the light morph
324 prevails. In fact, in its range in the Palaearctic the booted eagle has a clinal
325 polymorphism, where the proportion of the dark morph gradually increasing towards

326 the east, from approximately 19% of the Iberian Peninsula to 92% in the Altai-Sayan
327 mountains, presumably caused by climatic factors and different light conditions, which
328 exert a differentiated selective pressure on the two different morphs (Bosch 2019). Even
329 so, future behavioural and genetic studies should analyse the genotypes of adults and
330 offspring and their heterozygosity levels (e.g. Ortego et al. 2009; Gangoso et al. 2011;
331 Bourgeois et al.2017) to elucidate the mechanisms of mate choice and polymorphism
332 maintenance in this species.

333

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345

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519

520 **Table 1** Pair types, number of expected and observed breeding events, colour morph
 521 and morph ratio of the fledged eaglets in the three booted eagle populations studied in
 522 Spain (1995-2018).

Male-female morphs	Genotypes pairs	Breeding events		Eaglets fledged		Light/dark eaglets ratio	
		Expected	Observed	Light	Dark	Expected	Observed
Light-Light	LL x LL	112					
	LL x Ld	122					
	Ld x LL	77	393	610	5	17.75:1	122:1
	Ld x Ld	84					
Light-Dark	LL x dd	33	58	38	53	3.89:1	0.72:1
	Ld x dd	23					
Dark-Light	dd x LL	13	30	29	15	2.84:1	1.93:1
	dd x Ld	15					
Dark-Dark	dd x dd	4	2	0	3		
Total		483	483	677	76		

523

524

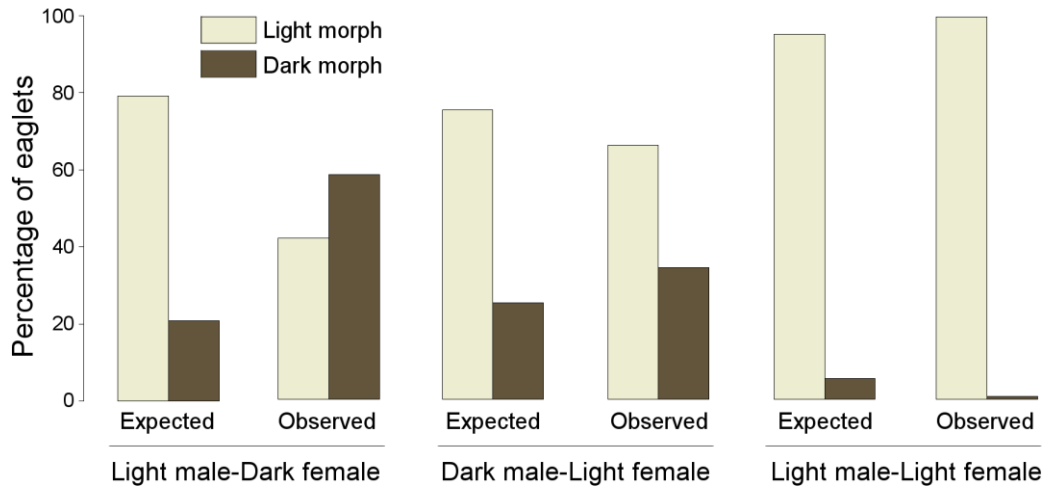
525 **Fig. 1** Location of the study areas: Central Catalonia (1), Els Ports Natural Park (2) and
526 Murcia province (3). Source: Stamen cartography(<http://maps.stamen.com/>), drawn in R
527 version 3.5.2 (<https://www.R-project.org/>) using the *rosm* package [https://CRAN.R-](https://CRAN.R-project.org/package=rosm)
528 [project.org/package=rosm](https://CRAN.R-project.org/package=rosm)).



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531 **Fig. 2** Expected and observed numbers of light-dark morph eaglets born from breeding
532 pairs of different male and female phenotypes in three booted eagle populations in
533 Spain (1995-2018).



534