

1 **Mating system, breeding success, and pup mortality of a habitat specialist rodent: a field and**
2 **molecular-based approach**

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29

30 **Abstract**

31 Mating systems are studied due to their interest in ecology and evolution. In rodents, mating strategies
32 have been inferred from the spatio-temporal arrangement of males and females, and breeding success has
33 usually been estimated through the number of embryos counted by palpation of pregnant females.
34 However, these might not be trustable proxies to describe the mating systems and to estimate survival rate
35 of pups. In this study, we surveyed breeding and reproduction of the southern water vole (SWV)
36 (*Arvicola sapidus*) in six ponds over a three-year period. We combined capture-mark-recapture data with
37 parentage analyses based on 444 individual genotypes with seven microsatellites i) to estimate
38 reproductive rate, ii) to compare field-based and molecular estimates of the mortality rate of juveniles and
39 the effective breeding success of adults, iii) to find individual traits or environmental conditions related to
40 breeding success, and iv) to describe the mating system of SWV. Our results suggest that mortality rate
41 during the pre- and post-weaning period is around 63% and 27%, respectively, and reinforce our
42 hypothesis that the high reproductive rates in SWV compensate for the high mortality of juveniles.
43 Parentage analyses assigned one or two parents to 80% of the target juveniles and suggest promiscuity as
44 the mating system of SWV, probably as a strategy to minimize infanticide by males, although deviations
45 from promiscuity might arise depending on variations of ecological factors. Weight is positively
46 correlated with reproduction rate, as heavier (dominant) females allocate nests in better habitat patches
47 (minimizing nest predation) and heavier (dominant) males expand their home ranges so they can have
48 access to more mates.

49

50 **Keywords:** southern water vole; *Arvicola sapidus*; microsatellites; litter size; promiscuity; pup mortality.

51

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57 **Conflicts of interest/Competing interests**

58 The authors have declared that no competing interests exist.

59 **Ethics approval**

60 The authors manipulated and marked Southern water voles approved by the Junta de Andalucía
61 Consejería de Medio Ambiente and the Estación Biológica de Doñana under permits linked to project
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63 **Consent to participate**

64 All authors agreed to participate in the project and preparation of the manuscript

65 **Consent for publication**

66 All authors agreed to publish this work.

67 **Availability of data and material**

68 Microsatellite dataset is available through the Mendeley Data Repository
69 (<http://dx.doi.org/10.17632/bxfhwft9xd.1>)

70 **Code availability**

71 Filters of the capture-mark-recapture databases in hosted Structured Query Language (SQL) with a
72 custom PYTHON script were written by María Lucena-Pérez and published in Lucena-Perez M, Soriano
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75 **Authors' contributions**

76 Conceived and designed the experiments: AC-C JR MD JAG. Performed the experiments: AC-C JR.
77 Analyzed the data: AC-C JR A.S-R M.L-P. Wrote the paper: AC-C JR AS-R ML-P MD JAG.

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79

80 Introduction

81 The mating system of a species or population is the outcome of breeding strategies of individuals to
82 maximize their fitness (or breeding success) (Waterman 2007). In animals, there are three mating systems
83 frequently described: monogamy, when only one female and one male mate; polygamy, named polyandry
84 when one female mates with several males, and polygyny when one male mates with several females; and
85 promiscuity, when each female mates with several males and each male with several females. Additional
86 attempts to classify mating systems including time scale have been recognized. For instance, an adult may
87 have a single mate during a breeding period but replace it over the course of its lifetime (sequential
88 monogamy or serial polygamy) (Wickler and Seibt 1983).

89 Molecular techniques have greatly contributed to a better comprehension of mating strategies, allowing
90 the discovery of hidden behaviors. In this way, whereas field studies suggested that nearly 90% of bird
91 species were monogamous (Lack 1968), the application of molecular markers to parentage analysis
92 showed that only 10% of species were in fact monogamous (Griffith et al. 2008). Identifying parental
93 pairs and classifying their progeny into full/half sibs is, therefore, key to determine the mating system of
94 species and contribute to a better understanding of the breeding success of individuals. A set of
95 sufficiently informative molecular markers together with likelihood and Bayesian statistical methods are
96 applied nowadays to assign parental pairs to offspring (Duchesne et al. 2005; Kalinowski et al. 2007;
97 Jones and Wang 2010) or to group individuals of a cohort into full- or half-sibships (Kalinowski et al.
98 2006) (reviewed in Flanagan and Jones 2019).

99 Mating systems and breeding success are conditioned by many internal and external factors
100 (phylogenetic, phenotypic, environmental, demographic...). Rodents provide a leading model system for
101 the study of the origin and, especially, the maintenance of mating systems in ecological and evolutionary
102 contexts (Waterman 2007). Understanding mate choice contributes to our knowledge of aspects of mating
103 systems, which are related to inbreeding avoidance through kin recognition or maximizing offspring
104 quality through the choice of unrelated or high quality mates (e.g., Lehmann and Perrin 2003). For this
105 reason, the study of behavior (Ims 1987), ecology (Morris 1989; Ostfeld 1990), and body condition
106 (Rémy et al. 2011; Godsall et al. 2014), including sexual dimorphism (Boonstra et al. 1993), has greatly
107 improved our knowledge on mating system in rodents.

108 Mating strategies in rodents have been studied for decades, mostly by observational techniques, leading to
109 the identification of monogamous (e.g., *Microtus ochrogaster*; Getz et al. 1981; *Iberomys cabreræ*;
110 Ventura et al. 1997), polygynous (e.g., *Microtus californicus*, *Microtus xathognatus*; Wolff 1985) and
111 promiscuous species (e.g., *Microtus pennsylvanicus*; Wolff 1985). However, as stated above in relation to
112 birds, observed reproductive behavior might mask the real mating system. The evolution and maintenance
113 of mating systems are the outcome of a conflict of interest between males and females, especially in terms
114 of their investment in the offspring through parental care and/or energy expenditure (eggs/sperm
115 production). It is commonly accepted that females benefit from monogamy (facilitating parental care
116 from their mate) and males do from polygyny or promiscuity (so they increase their breeding success).
117 However, deviations from these mating systems could be favored by the interaction between phylogeny,

118 ecological factors (e.g., resource availability), mate distribution, and mating dynamics (Clutton-Brock
119 1989; Klug 2018).

120 The southern water vole (hereafter SWV) (*Arvicola sapidus*) (Cricetidae, Rodentia) is a medium-size
121 (average adult weight in Doñana, females: 160 g, males: 166 g) arvicoline species considered as habitat
122 specialist due to its exclusive association to small patches of soft substrate and high vegetation cover
123 along water bodies (Román 2010). It is catalogued as “Vulnerable” in the Red List by the International
124 Union for Conservation of Nature (Rigaux et al 2008). Usually, SWV lives close to rivers and permanent
125 lagoons and ponds, but it has adapted, in terms of both habitat requirements and life history traits, to
126 occupy temporary ponds characterizing Mediterranean areas (Román 2007). In our study area (the
127 Doñana region, see below), rainfall is relatively high during the winter but completely stops during the
128 summer, creating scattered temporary water bodies in whose vegetation SWV settles its colonies. Field
129 and genetic studies in the area demonstrated that despite SWV being a habitat specialist, it shows a
130 generalist behavior during dispersal, as a response to its naturally fragmented breeding habitat (Centeno-
131 Cuadros et al. 2011)

132 Our first aim in this study is to unravel the mating system of SWV in the area. Previously, Román (2007)
133 studied individual movements of the species through capture-mark-recapture and suggested that bigger
134 females establish their territories on patches with the best habitat quality (i.e., colonies located at the
135 highest and more dense vegetation cover). Males, on the other hand, move across the colony trying to
136 include several females within their home range. By doing so, males often overlap their territories and
137 females have access to different mates. This scenario suggests promiscuity as the most likely mating
138 strategy, but this hypothesis has not been tested appropriately yet.

139 Our second aim is to estimate the breeding success of each parent discounting the losses of pups at birth
140 and during the first weeks of life. Usually, reproductive parameters of rodents (number of births per year,
141 litter size, etc.) have been estimated by counting embryos in dead or alive pregnant females (e.g.,
142 Catzeflis et al. 2019). If nest mortality is high, however, counts of embryos could severely overestimate
143 reproductive rates. Here, we assess productivity of adult SWV females using two methods: counting
144 embryos through palpation of pregnant females and using molecular markers to quantify the numbers of
145 postweaning juveniles assigned to each potential parent. By comparing both approaches, we can estimate
146 pup mortality and what might be called effective breeding success (i.e., a measurement of the number of
147 weaned juveniles per parent).

148 Last, we evaluated the effect of body size and body condition on the reproductive success of males and
149 females, respectively. In males, body weight seems to be related to dominance hierarchy so they can
150 access more females (Emlen and Oring 1977; Clutton-Brock 1989; Zedrosser et al. 2007; Clutton-Brock
151 and Huchard 2013); in females, a better physical condition increases fertility and litter size (Keller and
152 Krebs 1970; Ventura et al. 1986, 1990; Ribble 1992; Garde and Escala 1996; Riley et al. 2006; Correa et
153 al. 2016). For this reason, we tested whether weight had a positive and significant effect of on effective
154 breeding success. Additionally, many SWV individuals showed colored cutaneous lesions around the
155 genitalia attributable to poxviruses (authors, unpublished), and we predict that infection by poxviruses

156 will reduce individual breeding success. Finally, we hypothesized a negative effect of individual
157 (observed) homozygosity on life-long reproductive success measured as the total number of offspring per
158 reproductive individual (Huisman et al. 2016).

159 In summary, we specifically i) estimate reproductive rate by using field and molecular based approaches,
160 ii) compare these two approaches to gain insights about mortality rate of juveniles and the effective
161 breeding success of adults, iii) correlate breeding success with sex, body condition, individual
162 homozygosity and viral infection, and iv) define the mating system of SWV.

163

164 **Material and Methods**

165 *Study system*

166 The southern water vole was studied in the Doñana Natural Region, SW Spain (37° 10' N, 6° 23' W) (Fig.
167 1), between February 2000 and July 2002. Located at sea level, this area has a Mediterranean climate and
168 an average rainfall of 600 mm between October and May and a summer drought with no precipitation
169 from June to September. The rains lead to the formation of about 600 water bodies that SWV uses to live
170 along their shores (Fedriani et al. 2002; Román 2007). The whole breeding season usually extends from
171 October to May (potential breeding season), although there are two reproductive peaks related to the two
172 rainfall maxima that define the two breeding periods analyzed in this study: from February to May
173 (hereafter named “spring”) and from October to January (termed “autumn”) (see “Candidate parents and
174 scenarios” and Fig. 2). No breeding takes place in the summer drought season, when the voles retract to
175 small patches with particularly high and dense vegetation (Román 2007). Even during the rainy season,
176 only 2% of the total surface area is inhabited by the SWV, so SWV is obliged to survive within relict
177 habitat patches, especially during the drought season (Fedriani et al. 2002). However, landscape genetics
178 reveal that SWV shows high dispersal rates and long distance dispersal as an adaptation to colonize
179 distant habitat patches embedded within an unsuitable landscape matrix (Centeno-Cuadros et al. 2011).
180 According to Román (2007), SWV lives in colonies of six to 31 individuals (19.2 ± 8.18 , average \pm SD)
181 located along the water bodies. Males disperse more frequently than females (14.1% and 3.5%,
182 respectively), although dispersal distances do not differ between sexes (males: 838 m; females: 695 m).

183 Fieldwork and sampling were focused on six ponds (Carrizosa, Lucena, De la Res, Pino Quemado,
184 Moguer, and Sevillano) (Fig. 1) (Pond surface: 1.5 ± 0.6 ha (mean \pm s.d.; range: 0.7-2.5) (Table 1). Four
185 of these ponds were visited periodically (i.e., three trapping sessions of two days per potential breeding
186 season), starting as a pilot survey in Carrizosa and Lucena in Spring 2000 (20 trapping sessions overall)
187 and from Autumn 2001 from De la Res and Pino Quemado (16 trapping sessions overall). Moguer and
188 Sevillano were visited since Autumn 2001 only once for four and two breeding periods, respectively (Fig.
189 2). Trapping was optimized in Román (2007) and summarized as follows. Once fresh signs of SWV were
190 identified, crossroads and surroundings of the meadows were baited with oranges and apples. After two
191 days of prebait (and once bite marks of *Arvicola* were found on the orange rind), hand-made traps
192 (equipped with fresh bait and grass inside) were arranged at sunset and visited at sunrise. Newly captured

193 individuals were weighed, measured, ear-punched for genetic analyses, and individually tagged with a
194 microchip, whereas recaptures were weighed, recorded, and immediately released. Pregnant females were
195 always palpated to estimate the number of embryos. All individuals were live released and samples were
196 stored at 4°C in 95% ethanol and EDTA 100 µM. We classified individuals into their age categories
197 based on weight as this trait distinguished between juveniles (i.e., not sexually matured individuals) from
198 adults (sexually matured) within a 95% confidence interval in a logistic regression (females: weight of
199 juveniles, <94 g; adults, >123 g; males: juveniles, <100 g, adults, >130 g). A third age-class (subadults)
200 was defined (females: 95-122 g; males: 101-129 g) for those individuals whose weight exceeded that of
201 the juveniles but did not reach the minimum threshold value to be considered an adult (Román 2007). As
202 these individuals might be sexually mature, we performed parentage analyses including and excluding
203 subadults (see “Candidate parents and scenarios” for data analyses). Overall, 277 adults, 75 subadults,
204 and 92 juveniles were trapped (n=444, see Table 1) and classified into each of the five breeding periods
205 analyzed in this study according to their age and sex (see Online Resource 1). We considered that those
206 that were captured during fieldwork survived nest predation (among other mortality causes).
207 Demographic analyses revealed that ca. 85% of the living individuals in a pond at each trapping session
208 were captured (Román 2007). Each individual was classified according to the visible prevalence of a
209 poxvirus into categories from 0 (no infection) to 3 (maximum infection) by the same observer.

210

211 *Estimates of the number of pregnancies and litter size.*

212 Number of pregnancies and litter size rely on detecting and counting embryos in pregnant females. The
213 number of pregnancies was estimated based on a dataset of females (n=100) trapped within the
214 intensively surveyed ponds and it was used to estimate the frequency of pregnancy per observed breeding
215 season (g , see below). The number of pups per litter was mainly estimated by abdominal palpation of
216 every pregnant female captured from the survey in Román (2007), performed within Doñana but
217 extending to a broader area than the intensively monitored ponds included in this study. We also counted
218 the number of pups in seven occasions in which females delivered within the traps or while handling them
219 during field work.

220 We estimated the number of pregnancies per female following the equation $F=g*(T/v)$ by Emlen and
221 Davis (1948), where F is the average number of pregnancies a female has during each breeding season
222 (we assume it is equivalent to the number of births), g is the frequency of observed pregnancies
223 (gestations) in overall captured females, T is the average length (in days) of the breeding season measured
224 in this study (observed breeding season) (T may differ from the potential breeding season depending on
225 environmental conditions –mainly rainfall-), and v is the time span (in days) at which gestation is
226 perceptible. None of these parameters have been previously studied in SWV so we used our field
227 observations for g and T , and applied an estimation of v obtained from other rodent species.

228 We estimated the frequency of pregnancies (g) based on the number of observed pregnant females over
229 one hundred trapped females (adults and subadults) within the intensively monitored ponds in this study.

230 The observed breeding season (T) was defined as the time period in which pregnant females and/or pups

231 were trapped in any of the monitored ponds. We assumed that the detectability of embryos by palpation is
232 possible only during the last ten days of pregnancy ($v= 10$), as observed in other rodent species (Cole and
233 Batzli 1978; Stehn and Jannett 1981) and justified as follows. The duration of pregnancy is unknown in
234 SWV, but it seems a conserved trait in arvicoline species (Innes and Millar 1994) (20-24 days in *Microtus*
235 in North America (Nadeau 1985); 21-22 days in *M. arvalis* and *M. agrestis* (Ranson 1941; Pelikán 1982);
236 20-22 days in *A. terrestris* in Great Britain (Corbet and Southern 1977; Blake 1982) and Russia
237 (Nasledova et al. 1984). Consequently, we consider the time of pregnancy of *A. sapidus* around 21 days.
238 To our knowledge, there are no previous studies in SWV estimating the minimum size of embryos to be
239 detected by palpation. Nadeau (1985) suggested that growth patterns of embryos is conserved in rodents
240 and it is characterized by the implantation of embryo on the fifth day after copulation followed by a slow
241 development at early stages until day 10 (inclusive), when the growth of embryo accelerates. For this
242 reason, considering time of pregnancy of 21 days and an increased growth rate of embryos since day 11
243 of gestation, we assumed that the detectability of embryos by palpation is possible only during the last ten
244 days of pregnancy ($v= 10$).

245

246 *Parentage analyses*

247 All individuals were genotyped with a set of seven microsatellites [AV3, AV4, AV8, AV9, and AV15
248 (Stewart et al. 1999); AV10-2 (Centeno-Cuadros et al. 2011); Moe7 (van de Zande et al. 2000)] selected
249 based on their Polymorphic Index Content (PIC=0.778) and following the procedure described in
250 Centeno-Cuadros et al. (2011). We ran parentage analyses based on a maximum likelihood approach to
251 reconstruct pedigrees based on individual genotypes as implemented in COLONY 2.0 (Jones and Wang
252 2010). We run COLONY 2.0 to find the most likely pair of parents for each juvenile ($n=92$) across the six
253 sampled ponds using the allelic frequencies per pond from the genotypes of adults and juveniles estimated
254 in GENEPOP on the web (Rousset 2008). Parentage analyses were run using a dataset composed by
255 filtered candidate parents described as follows.

256 *Candidate parents and scenarios*

257 COLONY uses maximum likelihood to assign parentage relationships between target offspring and
258 candidate parents' sets. This analysis is sensitive to the number of candidate parents (Jones et al. 2010) so
259 we filtered our dataset to exclude candidates clearly unrelated to the target juvenile. First, we classified
260 individuals according to their age based on their weight (see above). This classification was used to run
261 parentage analyses per pond and for different datasets built according to the scenarios described below.
262 By doing so, we aimed to include the minimal number of candidate parents (including the real parents) to
263 increase the power of the assignments and avoid false assignments.

264 Preliminary results showed no change in parentage assignments when subadults were included in the
265 analyses as candidate parents, so we included both adults and subadults in further analyses. The scenarios
266 were defined by the breeding period where candidate parents were trapped (spring and autumn, see Fig.
267 2). According to the timing of sampling, Closed scenario included as candidate parents only adults that

268 were trapped (or recaptured) in the same trapping session than the juvenile under evaluation, while Open
269 scenario included also individuals that were trapped in a trapping session previous to the first capture of
270 the target juvenile (but not necessarily on the same trapping session). Both Closed and Open scenarios
271 yielded similar proportions of assignments (either paternity, maternity, or both parents were equally
272 assigned to the same juvenile in both scenarios), except in those cases where the assigned parent was not
273 trapped at the same trapping session than the juvenile (and, consequently, these candidate parents were
274 not included in the analysis of the closed scenario). In order to maximize the number of parental
275 assignments in this study we fused the outputs of both Open and Closed scenarios (Converged), but report
276 all results to increase the transparency of our analyses. Consequently, we generated different datasets per
277 pond using the Open, Closed and Converged scenario that we analyzed in five breeding periods between
278 February 2000 and July 2002 (Fig. 2). Candidate parents were selected for paternity/maternity
279 assignments based on filters of the capture-mark-recapture databases in hosted Structured Query
280 Language (SQL) with a custom PYTHON script (Lucena-Perez et al. 2018). Parentage analyses were then
281 run in COLONY and output files were explored using R (The R Core Team 2016) and *Dplyr* (Wickham
282 and Francois 2016).

283 Results obtained in COLONY were used to identify littermates (i.e., set of juveniles sharing the mother in
284 the same gestation). Since input files per pond and breeding period may include juveniles of different
285 ages (days) (e.g., juveniles from different mothers and litters and/or offspring sharing mother but from
286 different litters within the same breeding period), we used Román (2007) to estimate the age of juveniles.
287 By doing so, we discarded as litters those composed by full/half sibs of different ages. We then classified
288 litters into single and multiple (paternity) if they were composed by full or half sibs (respectively).

289

290 *Statistical analyses*

291 We modelled the breeding success of adults using the results from parentage analyses as response
292 variable and assessed the potential effect of sex, weight, individual homozygosity (HL) and prevalence of
293 poxvirus (virus) to test the previously described hypotheses. We considered only adult individuals
294 captured at the four intensively monitored ponds (Carrizosa, De la Res, Pino Quemado, and Lucena) for
295 this analysis. We used the homozygosity by loci (HL) (Aparicio et al. 2006), an individual index of
296 homozygosity that weights the contribution of each locus by their allelic variability. HL ranges from 0 to
297 1 when none or all of the loci are homozygous (respectively). We assessed the effects of sex, weight, HL,
298 and virus on breeding success using Generalized Linear Mixed Models (GLMM) using the function *glmer*
299 implemented in *lme4* (Bates et al. 2015) in R. We transformed the number of juveniles assigned to each
300 (sub)adult to a response variable by fitting to a binomial distribution where breeding (1) or not (0)
301 depended on the fixed effects described above (sex, weight, HL, and virus). Pond and individual were
302 included in the model as random effects. The final model was selected using a backward selection
303 procedure and every model was compared with the null model (including only the intercept).

304 The microsatellite dataset generated and analyzed during the current study is available in the Mendeley
305 Data Repository (<http://dx.doi.org/10.17632/bxfhwft9xd.1>)

306 **Results**

307 *Using field data: observed breeding season, number of pregnancies per female, and litter size by*
308 *palpation of embryos*

309 The timespan of the observed breeding season of SWV ranged between five and nine months (this
310 difference is explained by the variance between ponds and years) (mean= 7; s.d.= 1.2) ($T = 7 \text{ months} = 7 * 30.5 = 214$ days) and reproduction was absent since early June until mid-October. The frequency of
311 pregnant females detected by palpation during an effective breeding season was $g = 0.17$.
312

313 The potential (estimated) number of pregnancies per breeding period was $F = 3.6$ births per adult female.
314 The estimated number of embryos counted by palpation was 3.3 ± 1.1 (average \pm s.d., $n = 62$) and it was
315 reinforced by the number of pups per birth observed during field work (3.3 ± 1.6 , $n = 7$) (Mann–Whitney
316 $U = 192.5$, $p = 0.992$). The mode was three ($n = 69$) and ranged between one and six pups per gestation.

317

318 *Using molecular-based parentage analyses: reproductive rate, surviving litter size, parental assignments,*
319 *and mating strategies*

320 The overall number of assignments (only female, only male, or both parents) per scenario are reported in
321 Table 2. The analysis based on the Converged scenario assigned 52.2% ($n = 48$) of the juveniles to both
322 parents and 15.2% ($n = 14$) and 11.9% ($n = 11$) to only the mother and only the father (respectively). These
323 results based on molecular methods describe parental assignments to juveniles that survived their
324 preweaning period and were trapped during fieldwork. Consequently, these values report the effective
325 breeding success that will later on be contrasted with the estimated number of pregnancies per female and
326 embryos/offspring per litter based on field data. Due to our estimation of a recapture rate of 85% (Román
327 2007), we are confident that survival and litter size are not grossly underestimated by observed rates.

328 From the parent perspective, only 19 males (14.7% of the total number of adult males genotyped, $n = 129$)
329 sired any of the identified litters (see below) of which 11 bred only once. Four and two males bred with
330 two and three females, respectively (polygyny), and two males bred twice with only one female
331 (monogamy). Similarly, 23 females (15.5% of the number of genotyped adult females, $n = 148$) bred only
332 once ($n = 16$) or twice within the same ($n = 6$) or different ($n = 1$) breeding periods (but females losing the
333 whole litter at the nest would not be considered; see below). A single offspring was assigned to six
334 females, and 15 females had more than one offspring per litter, which helped to identify single or multiple
335 paternity. Parental assignments of these litters with two or more juveniles revealed that 70% of females
336 bred with a single male (monogamy) whereas 30% bred with two males (polyandry). All seven females
337 breeding twice mated with two different males in each reproductive event (serial polyandry). Only one
338 female bred in two non-consecutive breeding periods (spring 2001 and spring 2002; this female did not
339 breed in autumn 2001). The highest sum of juveniles inferred by parental assignment per male and female
340 were six and seven, respectively.

341 From the litters' perspective, parentage analysis grouped 73 juveniles in 30 litters (number of juveniles
342 per litter: 2.4 ± 0.89 , mean \pm SD) (min=1, max=4) (see Converged scenario, Table 2), understood here as
343 the group of juveniles of the same age that share the same mother. As indicated, these estimations refer to
344 "successful" litters, that is, littermates that survive the weaning period. We assigned 12 juveniles only to
345 their father or mother (the other parent remaining unknown, hereafter, half-assigned juveniles) and
346 grouped juveniles into 18 litters composed by two or more siblings. When juveniles were grouped in
347 litters, they were either full-sibs (single paternity litters, n=9) or half-sibs (multiple paternity litters, n=9).
348 Litters were classified as "multiple paternity" when parentage analyses assigned one mother and two
349 fathers to a group of littermates (type I, n=4), when at least one juvenile within the litter was not assigned
350 to any candidate male (type II, n=1) or when two different unidentified males fathered the litter (type III,
351 n=4).

352 The rate of parental assignments differed among ponds (Fig. 3). The rate of assignment of both parents
353 ranged from 77.8% (eight out of nine juveniles in De la Res) to 25% (two out of eight in Sevillano). The
354 rate of single parent assignments was very different for male and female parents: the "only father"
355 assignments (mother remained unknown) were scarce or even null (only in Moguer and Sevillano, the
356 two less intensively sampled ponds), whereas "only mother" assignments (father remained unknown)
357 were obtained in all ponds except De la Res.

358

359 *Breeding success, life traits, and body condition*

360 Only weight had a positive and significant effect on breeding success (GLMM: $Z = 3.085$, $p = 0.002$; Table
361 3 and Fig. 4). The best model was selected using the Akaike Information Criterion (AIC) after removing
362 non-significant variables (and their interactions) ($AIC_{\text{null}} = 229.3$; $AIC_{\text{weight}} = 226.5$). Weight had an effect
363 of 60% on breeding success and the probability of prediction was estimated on 92% ($IC_{95\%} = (80.77\% -$
364 $97.78\%)$) (significance level, $\alpha = 0.05$). According to the selected model, individuals of average weight
365 (189 g) had a probability of reproduction of 9.66% whereas this probability ranged from 3.63% to 31.43%
366 in individuals of the lowest (131 g) and the highest (247 g) weights, respectively.

367

368 **Discussion**

369 Although the reproduction of SWV has been one of the most studied aspects of its biology (Ventura et al.
370 1986, 1990; Garde and Escala 1996), little is known about individual strategies to maximize fitness.
371 Moreover, even though promiscuity has been suggested as a common reproductive strategy (Román
372 2007), this hypothesis was not tested previously. The combination between intensive field and molecular
373 data and the statistic approach implemented in this study made it possible to unravel the breeding success
374 and mating strategies of SWV in patchy and heterogeneous habitats, and led to estimates of the breeding
375 success of males and females and the mortality of juveniles (before and after weaning). Below, we
376 discuss why the number of pregnancies a female has on average differed from the actual number of litters

377 assigned using molecular methods and, moreover, why the number of offspring a female delivered (i.e.,
378 palpated in the field) differed from the number of offspring assigned.

379

380 *Reproductive rate and effective breeding success in the southern water vole*

381 Southern water voles are habitat specialist rodents adapted to survive within patchy and heterogeneous
382 habitats such as those occurring in the Natural Area of Doñana. The environmental conditions and
383 population dynamics of the species restrain the average life expectancy of males (3.8 months) and
384 females (5.2 months), only 6.9% and 15% of males and females (respectively) surviving longer than a
385 year (Román 2007) (in this study, we have only documented one female breeding in more than one
386 breeding period). During this short lifespan, field data revealed that females breed an average of 3.6
387 times, whereas breeding success of males was unknown mainly due to their breeding behavior. The
388 molecular approach implemented in this study reveals that the proportion of parenting females and males
389 is relatively low but similar (15.5% and 14.7%, respectively) reducing the mean observed (field based)
390 number of 3.6 embryos to 0.54 juveniles per female. The comparison between the number of embryos per
391 female (counted by palpation) and the number of (surviving) offspring (inferred with molecular parentage
392 analyses) has provided insights on breeding success of adults and mortality rates in pups and juveniles.
393 Parental assignments are focused on juveniles that were trapped after weaning and, therefore, have
394 survived mortality within nests. Our results, therefore, reveal a frequent loss of entire litters, which might
395 be explained by nest predation and/or infanticide, presumably by males (although predation of some
396 siblings from the same litter was also described, see half-assigned juveniles, above). This might explain
397 why some pregnant females trapped during fieldwork were not assigned as mothers to any of the
398 juveniles in the parentage analyses. A correction to avoid such bias would be sampling within nests and
399 always before predation, or sampling and genotyping embryos of pregnant females (e.g. Miller et al.
400 2010).

401 What are the individual traits leading breeding success? Our results have revealed that individuals might
402 increase their probability of reproduction up to ten times (3.63% to 31.43%) depending on their weight,
403 which is ultimately related to physical condition and/or age (see Fig. 4). Román (2007) found this effect
404 in females, as heavier females were dominant and selected habitat patches of high quality (high
405 vegetation cover and density) compared to subordinated females. Variations in food availability and
406 environmental conditions might alter body size of females, number of offspring per litter, and their
407 survival rate (King et al. 1991; Koskela 1998), although we did not test experimentally this hypothesis in
408 this study. Dominance in females, therefore, is important not only because of food availability, but also
409 because habitat quality regulates the susceptibility of nests to predation, so it will ultimately influence
410 individual breeding success. This is clearly illustrated by the comparison of the number of pregnant
411 females observed in the field with the inferred proportion of parenting females. Consequently, a better
412 physical condition (mirrored by weight) increases the chances of reproduction of both sexes: heavier
413 females establish their home ranges in better habitat quality patches, whereas body weight rules

414 dominance in males and, consequently, their access to other females (the bigger their home range, the
415 higher their access to other females).

416

417 *Litter size and effective breeding success*

418 The comparison between the number of embryos and the number of offspring assigned to a female
419 contribute to a better understanding of perinatal mortality. We stress that parentage analyses using
420 molecular tools estimate the so-called effective breeding success and must be differentiated from the raw
421 reproductive rate of females. Molecular methods are based on genotypes of juveniles that have survived
422 nest predation and clearly underestimate the raw reproductive rate of females. The comparison between
423 these two estimates (average number of embryos per litter: 3.3; average number of juveniles per litter, as
424 inferred from parentage analyses: 2.4) suggest that mortality rate in juveniles (ca. 28%) is lower than
425 mortality rate of pups (nest predation and/or infanticide). But, how frequent is nest predation in SWV in
426 Doñana? A female has an average of 13 pups per observed breeding season (3.3 embryos/pregnancy and
427 3.6 births/breeding period) and the mean number of (surviving) juveniles inferred by molecular methods
428 is 2.4 juveniles per litter. Considering two as the maximum number of litters per female documented in
429 this study, we can extrapolate this result to 4.8 as the maximum number of (surviving) pups per effective
430 breeding season, that is, 27% of pups will survive and 63% will be likely predated within their nests. This
431 result shows how critical it is for females to set their home ranges in the best habitat quality patches at the
432 beginning of the breeding season to prevent nest predation, and emphasizes the effect of weight and
433 dominance on breeding success.

434 There is, however, a high variance in breeding success among individuals. Besides the low percentage of
435 breeding success (15.5% and 14.7% of females and males, respectively), 70% of these females bred
436 successfully a single litter, and 81% were single paternity litters and the remaining 19% litters of two
437 different males (see “Mating strategies of the southern water vole” below). We estimated that, among
438 individuals breeding at least twice, both females (n=5, 100%) and males (n=6, 75%) changed their mates
439 in consecutive reproductive events (polyandry and serial polygyny). Combining Capture-Mark-Recapture
440 methods with parentage analyses would allow relating the dispersive movements of individuals with their
441 reproductive success. Unfortunately, the low number of dispersers that bred in our study (two males and
442 one female) did not allow the comparison between the reproductive success of dispersers and non-
443 dispersers individuals.

444

445 *Mating strategies of the southern water vole*

446 We have shown that most of the litters of SWV are monogamous when considering the number of fathers
447 single litters have (single paternity litters: 81%; litters fathered by two males: 19%). However, the
448 analysis of these promiscuous mating events also suggests that both females (n=5, 100%) and males (n=6,
449 75%) changed their mates in consecutive reproductive events, which also proves polyandry and serial
450 polygyny in SWV. Mating system in SWV might be, therefore, controlled by some ecological factors

451 such as patch size and quality, competition, population, and/or sex ratio, favoring a shift from monogamy
452 to promiscuity across the whole gradient of mating systems variation (reviewed in Lott 1991).
453 Promiscuity is common in other habitat specialists rodents (McEachern et al. 2009; Sommaro et al. 2015)
454 in areas where resource distribution is patched and limited, as it happens, for instance, in the European
455 snow vole (*Chionomys nivalis*) (Luque-Larena et al. 2004). This species lives in isolated patches
456 distributed in rocky mountain areas where females compete for shelter and trophic resources and males
457 try to maximize the number of females within their territories. As in SWV, these conditions cause a large
458 overlap in their home ranges so males increase their chance to mate more females and, therefore, their
459 probability of multiple mating. Females, on the other hand, may use multiple paternity as a strategy to
460 minimize the probability of infanticide by males, as it has been suggested in the northern sister species
461 *Arvicola amphibius* (formerly *A. terrestris*) (Jeppsson 1986) (but see Frafjord 2016) and many other
462 vertebrates (Clutton-Brock and Huchard 2013). As Hrdy (1974) first suggested, by killing offspring they
463 have not sired, the perpetrator male causes the mother to become shortly receptive, with a good chance to
464 sire her next litter, and thus increasing his fitness. Confounding paternity by mating with several males is
465 a good counter-strategy of females (Agrell et al. 1998), although infanticide by females cannot be ruled
466 out. Moreover, it is also likely that pregnant females carrying offspring of multiple fathers might
467 counteract founder effects and inbreeding depression associated to a metapopulation dynamics such as
468 the one described in Doñana (Fernández et al. 2016). A single pregnant colonizer that carries alleles from
469 at least three founders at once (mother and at least two fathers) increases her chances to establish a new
470 population and increases survival of their progeny and their breeding success. This strategy has been
471 shown in invasive species such as *Rattus norvegicus* and *R. rattus* (Miller et al. 2010) and highlights the
472 relevance of multiple paternity for the viability of populations funded by single females (Russell et al.
473 2009).

474 Parentage analyses based on genetic markers are often limited by the incomplete sampling of candidate
475 parents. The exhaustive sampling and demographic analyses in Román (2007) confirmed that ca. 85% of
476 the individuals were trapped in this study. However, we could not assign any parent to ca. 20% of the
477 target juveniles (Table 2), which might be caused by unsampled adults and/or lack of power of the
478 molecular markers to unambiguously select the true parent. We could not differentiate whether
479 unsampled fathers were dispersers or adult males that died soon after copulation, or adult females that
480 died after weaning. In fact, high predation rates over SWV in Doñana have been reported, mainly by the
481 Egyptian mongoose (*Herpestes ichneumon*), genet (*Genetta genetta*), barn owl (*Tyto alba*), and black-
482 winged kite (*Elanus caeruleus*) (Delibes et al. 1984; Palomares and Delibes 1991; Román 2007).

483 Whichever the reason, the average net reproductive rate of 0.54 juveniles/females seem insufficient to
484 sustain the viability of the populations. Southern water voles in Doñana live close to the bound of their
485 potential ecological niche. Long-term monitoring of the species has revealed a population decline over the
486 last years that might be caused by a decline of habitat availability (related to droughts), water
487 exploitation, and livestock (Román 2007; Delibes and Román 2015; Fernández et al. 2016). Habitat loss
488 decreases the availability of areas with high vegetation cover so SWV are forced to locate nests in
489 suboptimal areas and causes the low breeding success and nest predation observed in this study.

490 Controlling these threats by e.g., decreasing groundwater exploitation and conserving peripheral
491 vegetation along water bodies by decreasing livestock pressure will help to maintain SWV populations in
492 Doñana.

493 In conclusion, we suggest that nest predation (including infanticide) in SWV causes a mortality rate of
494 63% of preweaning individuals, whereas 28% of juveniles might die during their first days once they
495 leave their nests. For this reason, the allocation of nests is crucial to ensure litter/pups survival and we
496 found weight (and, therefore, dominance) as the ultimate individual trait directly related to breeding
497 success: whereas dominant females choose better habitats, dominant males increase their home ranges
498 and, therefore, their chance of mating other females. SWV is a promiscuous species and both sexes
499 benefit from multiple paternity, although deviations from promiscuity might arise depending on variations
500 of ecological factors. We hypothesize that multiple paternity in females might be an adaptation to living
501 in patchy and heterogeneous habitats by reducing infanticide by males and increasing genetic diversity
502 (and, therefore, population survival) in new colonies. We are aware that some of our estimates, although
503 the best with the available data, might show any variance caused by the annual pattern of rainfall.
504 Nonetheless, we believe that our study contributes not only to increase our knowledge on *Arvicola*
505 *sapidus*, but also to the current methodology on the study of mating strategies and mortality at early age
506 stages. Further research must focus on survival rates and breeding success of dispersers vs. non-dispersers
507 to elucidate the benefits of dispersal on individual fitness and their effect on genetic diversity.

508

509

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659

660

661

662 **TABLES AND FIGURES**

663 **Table 1.** Individuals included in parentage analyses classified by ponds (rows) and their age classes
664 (columns).

665

666

667

	Surface (has)	Adults	Subadults	Juveniles	Total
668 Carrizosa	1.35	41	10	13	64
669 De la Res	2.50	31	7	9	47
670 Pino Quemado	1.01	59	21	11	91
671 Lucena	1.82	85	26	40	151
672 Moguer	0.53	47	8	11	66
673 Sevillano	1.72	14	3	8	25
674 Total	8.93	277	75	92	444

675

676 **Table 2.** Number and type of assignments resulting from parentage analyses at each scenario (see text for
677 explanation)

678

679

		Scenario			
		Closed	Open	Converged	% Converged
681	Complete	38	35	48	52.2
682	Only mother	12	13	14	15.2
683	Only father	9	18	11	11.9
684	No assignment	33	26	19	20.7

685

686 **Table 3.** Results of the GLMM using binomial distribution and logit link function. Pond and individual
687 ID were used as nested random effects.

688

689		Estimate	SE	Z	P
690	Intercept	-2.33835	0.38306	-6.104	1.03e-09
691	Sex	0.23375	0.36148	0.647	0.51785
692	Weight	0.60788	0.19702	3.085	0.00203
693	HL	-0.01635	0.19255	-0.085	0.93233
694	Virus	0.03603	0.15070	0.239	0.81104

695

696

697

698 **FIGURE CAPTIONS**

699 **Fig. 1** Study area in Doñana Natural Region (SW Spain). The six ponds where southern water voles were
700 sampled are highlighted in white (see Table 1 for pond surface and sample sizes)

701

702 **Fig. 2** Sampling scheme of southern water voles in Carrizosa, De la Res, Pino Quemado, and Lucena. The
703 breeding periods considered for the different scenarios for parentage analyses (see text) are also shown.
704 Months are encoded by numbers and ponds were visited twice each breeding period.

705

706 **Fig. 3** Proportion of parental assignments of southern water voles in each pond. Sample sizes per pond are
707 shown above each bar

708

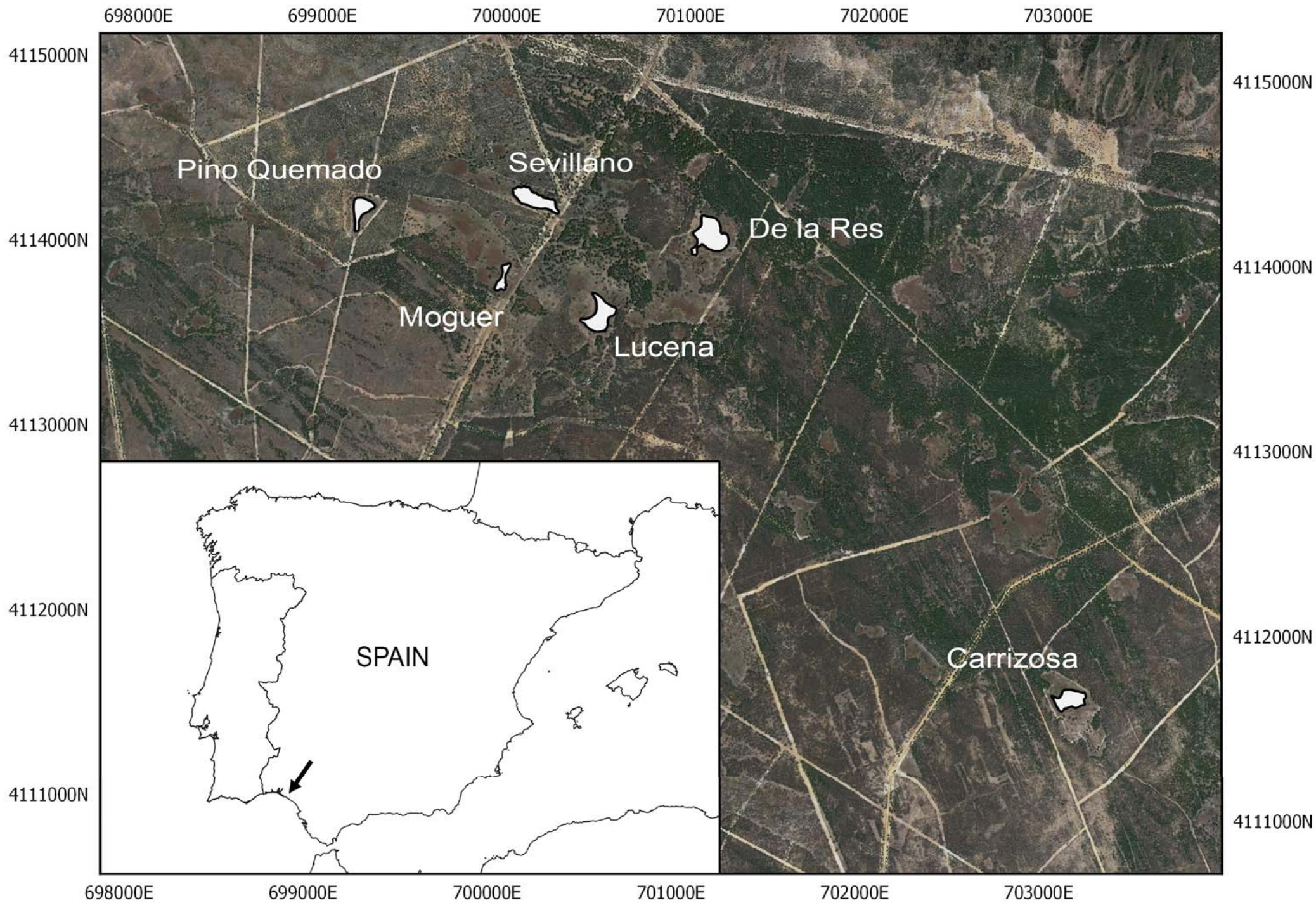
709 **Fig. 4** Effect of weight on the probability of reproduction of southern water vole (slope = 0.606)

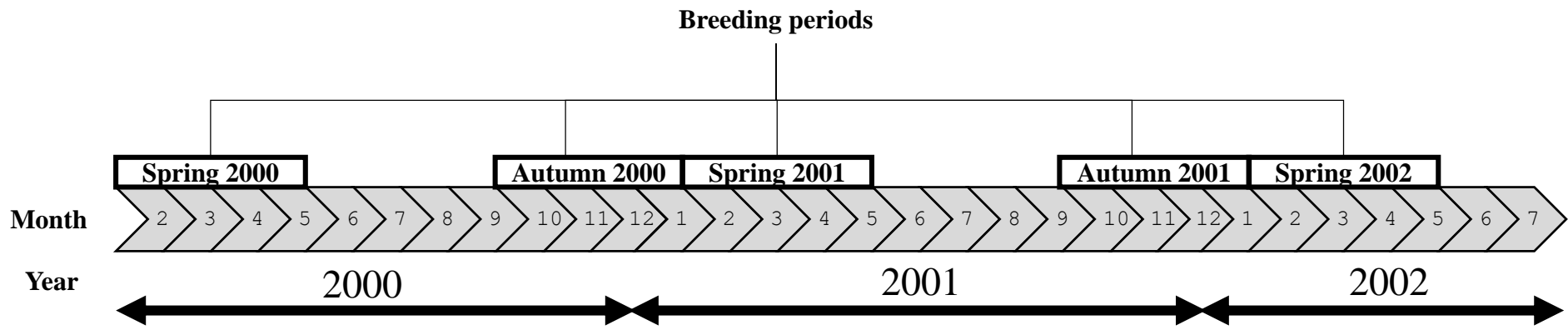
710

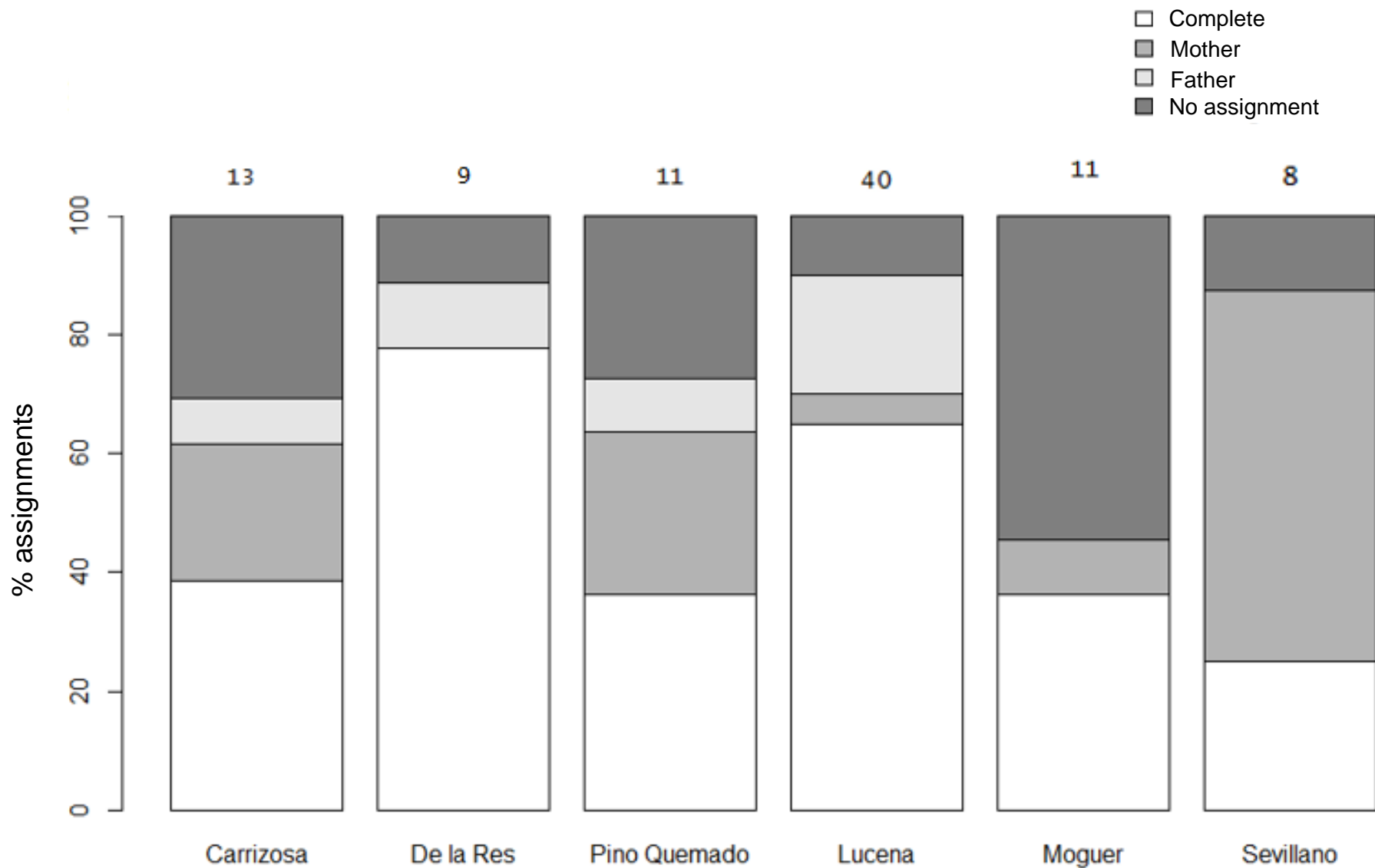
711 **ONLINE RESOURCES**

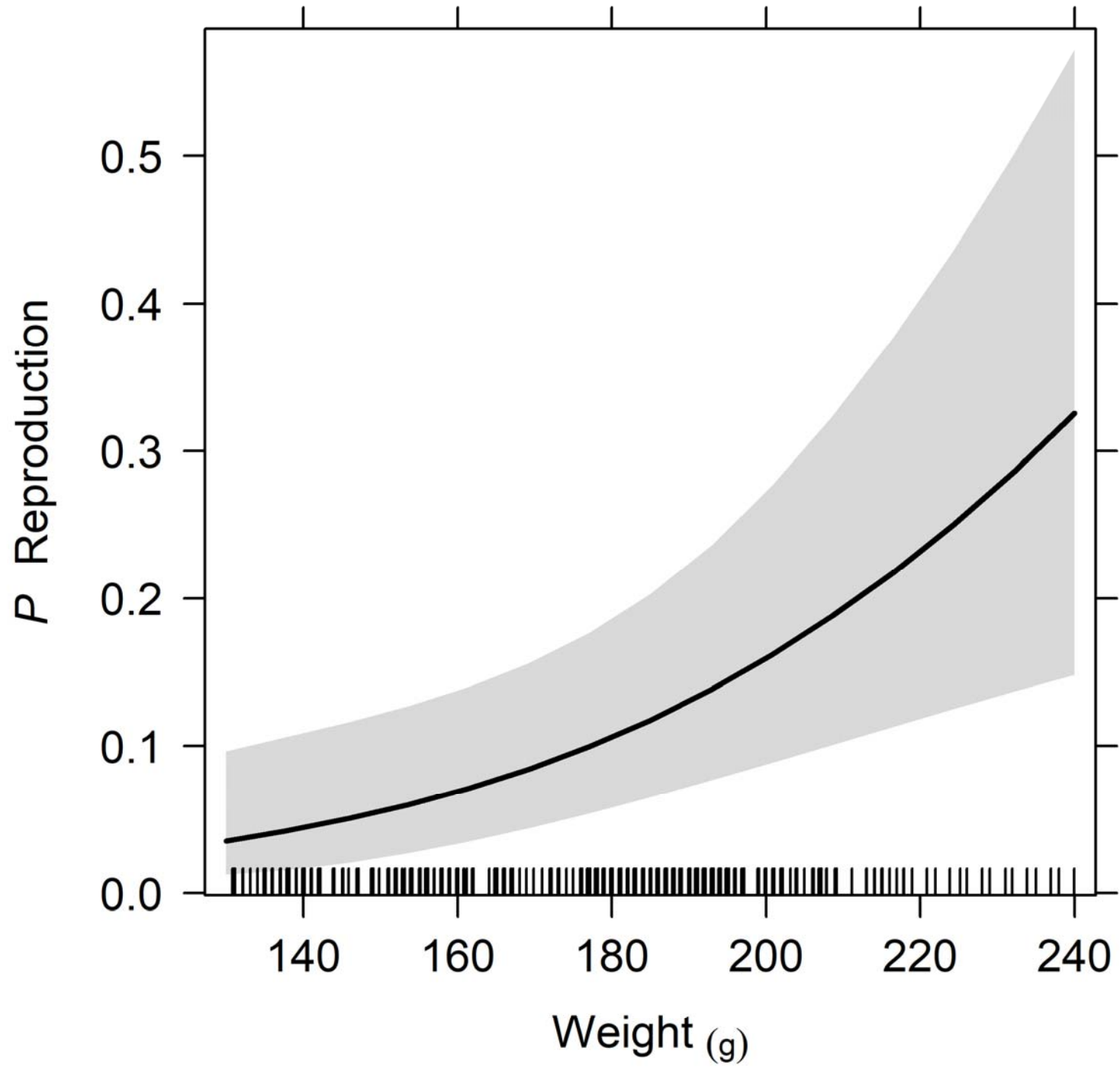
712 **Online Resource 1.** Sample size per age and sex of the southern water voles included in data analyses per
713 breeding period. F: females. M: males

714









ONLINE RESOURCE 1

Mating system, breeding success, and pup mortality of a habitat specialist rodent: a field and molecular-based approach

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Online Resource 1. Sample size per age and sex of the Southern water voles included in data analyses per breeding period. F: females. M: males

Spring 2000

	Adults		Subadults		Juveniles		Overall
	F	M	F	M	F	M	
Carrizosa	6	5	1	4	4	0	20
De la Res	0	0	0	0	1	0	1
Lucena	6	6	0	0	2	5	19
Moguer	0	0	0	0	0	1	1
Sevillano	0	0	0	0	0	1	1
Overall	12	11	1	4	7	7	42

Autumn 2000

	Adults		Subadults		Juveniles		Overall
	F	M	F	M	F	M	
Carrizosa	16	10	3	3	0	0	32
De la Res	8	7	3	2	0	0	20
Pino Quemado	14	13	7	4	0	0	38
Lucena	23	20	3	3	0	2	51
Martinazo	2	5	0	0	0	0	7
Moguer	2	2	0	0	0	0	4
Sevillano	3	7	1	0	0	0	11
Overall	68	64	17	12	0	2	163

Spring 2001

	Adults		Subadults		Juveniles		Overall
	F	M	F	M	F	M	
Carrizosa	18	12	3	4	4	5	46
De la Res	11	8	5	3	2	6	35
Pino Quemado	19	17	7	7	4	2	56
Lucena	27	19	7	9	9	4	75
Martinazo	2	5	0	0	0	0	7
Moguer	11	7	0	0	1	0	19
Sevillano	6	10	1	0	0	0	17
Overall	94	78	23	23	20	17	255

Autumn 2001

	Adults		Subadults		Juveniles		Overall
	F	M	F	M	F	M	
Carrizosa	10	9	2	3	0	0	24
De la Res	7	3	0	2	0	0	12
Pino Quemado	17	14	3	2	0	1	37
Lucena	24	16	5	5	0	0	50
Martinazo	2	5	0	0	0	0	7
Moguer	16	10	0	0	0	1	27
Sevillano	5	3	0	0	3	4	15
Overall	81	60	10	12	3	6	172

Spring 2002

	Adults		Subadults		Juveniles		Overall
	H	M	H	M	H	M	
Carrizosa	0	2	0	0	0	0	2
De la Res	8	2	0	0	0	0	10
Pino Quemado	23	15	5	2	2	2	49
Lucena	14	15	6	3	13	5	56
Martinazo	2	2	0	0	0	0	7
Moguer	16	15	2	6	3	5	47
Sevillano	2	2	2	1	0	0	7
Overall	65	53	15	12	18	12	178