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1 **Title**

2 Field study reveals morphological and neuroendocrine correlates of seasonal breeding in
3 female water voles, *Arvicola terrestris*.

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16

17

18 **Abstract (150 words)**

19 Seasonally breeding mammals display timely physiological switches between reproductive
20 activity and sexual rest, which ensure synchronisation of births at the most favourable time of
21 the year. These switches correlate with seasonal changes along the hypothalamo-pituitary-
22 gonadal axis, but they are primarily orchestrated at the hypothalamic level through
23 environmental control of KISS1-dependent GnRH release. Our field study shows that births
24 of fossorial water voles, *Arvicola terrestris*, are concentrated between March and October,
25 which indicates the existence of an annual reproductive cycle in this species. Monthly field
26 monitoring for over a year further reveals dramatic seasonal changes in the morphology of the
27 ovary, uterus and lateral scent glands, which correlate with the reproductive status. Finally,
28 we demonstrate seasonal variation in kisspeptin expression within the hypothalamic arcuate
29 nucleus. Altogether, this study demonstrates a marked rhythm of seasonal breeding in the
30 water vole and we speculate that this is governed by seasonal changes in photoperiod.

31 **Keywords**

32 Seasonal breeding; Field study; hypothalamo-pituitary-gonadal axis; Kisspeptin; Water voles;
33 *Arvicola terrestris*

34 1. Introduction

35 In seasonally breeding mammals, annual sexual activity is temporally restricted to allow
36 synchronised births when environmental conditions, such as temperature and food
37 availability, are most favourable to the survival of the juveniles and mothers (Vasantha,
38 2015). The annual cycle of sexual activity and sexual rest of seasonal mammals depends upon
39 major central regulations of the neuroendocrine axis which controls the reproductive function,
40 namely the hypothalamo-pituitary-gonadal axis (for review see Simonneaux *et al.*, 2013).
41 Two populations of kisspeptin neurons, respectively located in the anteroventral
42 periventricular nucleus (AVPV) and arcuate nucleus of the hypothalamus, play distinct but
43 pivotal roles in the central control of reproduction. Kisspeptin is the most potent secretagogue
44 of GnRH (Messenger *et al.*, 2005), which stimulates the release of FSH and LH in the
45 bloodstream by the gonadotropic cells of the pituitary gland (Belchetz *et al.*, 1978). In adult
46 females, FSH and LH act on ovaries to support follicular growth, allow ovulation and
47 formation of *corpora lutea* (Greep *et al.*, 1942) and trigger the production of the sex-steroids
48 estrogen and progesterone. In turn, sex steroids influence the growth of the uterus (Brody and
49 Wiqvist, 1961; Galand *et al.*, 1971) and secondary sexual characteristics, as well as sebaceous
50 glands (Frost *et al.*, 1973; Thiessen *et al.*, 1971). At least in rodents, estrogen exerts positive
51 feedback onto kisspeptin neurons located in the AVPV, which triggers the pre-ovulatory LH
52 surge, but negative feedback onto kisspeptin neurons of the arcuate nucleus, which is key for
53 the pulsatile secretion mode of LH (Gottsch *et al.*, 2009; Smith *et al.*, 2005). Furthermore, in
54 seasonal breeders, there is strong evidence that these Kiss1-expressing neurons of the arcuate
55 nucleus relay photoperiodic information and are crucial for the timely orchestration of
56 seasonal switches in reproductive states (Simonneaux, 2020)

57 Our understanding of the mechanisms regulating the HPG axis in temperate zone non-
58 hibernating fossorial species is currently limited. To address this gap, we have studied
59 *Arvicola terrestris*, which is the fossorial ecotype of the water vole, *Arvicola amphibius*, a
60 small semi-aquatic rodent species that displays widespread distribution across Eurasia
61 (Mahmoudi *et al.*, 2020; Shenbrot and Krasnov, 2005). Fossorial water vole mainly colonizes
62 mid-mountain permanent meadows and orchards where it displays a mostly underground
63 lifestyle. It digs extensive networks of galleries, which are used for foraging and storing food,
64 especially during autumn and winter, and it builds nests (Airoidi, 1976).

65 Births of water voles are mainly observed from March to October (Airoidi, 1978; Stoddart,
66 1971; Ventura and Gosálbez, 1990; Villette et al., 2020) with an average of three litters per
67 year. It has been hypothesized that the sexual activity of water voles displays an annual
68 rhythm, which has been empirically supported by the annual fluctuation in the activity of
69 androgen-dependent lateral scent glands of adult males. These sebaceous glands were
70 characterised by a hypertrophied epidermis and the absence of hair follicles and appear to be
71 implicated in the attraction of sex-partner and territoriality (Saucy, 1988; Stoddart, 1972;
72 Stoddart et al., 1975). However, the sexual activity of fossorial water voles may be
73 maintained during mild winters in some populations located in Switzerland (Meylan and
74 Airoidi, 1975). Likewise, when exposed to favourable weather conditions, as occurs in the
75 northwest of Spain, fossorial voles can display year-round breeding (Somoano et al., 2017). In
76 this context, the regulation of the annual rhythm of sexual activity of water voles needs
77 further investigation. Here, we explored the hypothesis that seasonal regulations, mediated by
78 kisspeptin, along the hypothalamo-pituitary-gonadal axis were responsible for the alternation
79 between periods of breeding and sexual rest.

80 To this end, we have performed monthly monitoring of trapped fossorial female water voles
81 from February 2019 through to June 2020 in order to characterise seasonal regulations of
82 breeding at different levels, i.e. along the hypothalamo-pituitary-gonadal axis and at the level
83 of the steroid-dependent lateral scent glands. We expected to observe, during the breeding
84 period, growth of genital organs (ovaries and uterus) and lateral scent glands compared to
85 winter. Furthermore, we anticipated that these changes would correlate with changes in
86 kisspeptin immunoreactivity in the hypothalamic arcuate nucleus.

87 **2. Material and methods**

88 2.1. Ethical statement

89 The trapping of water voles was authorised in the French department of Puy-de-Dôme by
90 prefectural authorization 19-00100. This study was carried out in accordance with European
91 directive 2010/60/UE and was approved by an ethical committee for animal experimentation
92 (C2EA-02, project 21994-201907510411944).

93 2.2. Study species

94 The European water vole belongs to the Cricetidae family, which also includes voles of the
95 genera *Microtus* and *Myodes* and hamsters (Wilson and Reeder, 2005). The fossorial ecotype
96 of water voles in the study area (Auvergne, France) has an adult weight of 75-160g and
97 approximately measures fifteen centimetres, twenty with the tail. The reproductive capacities
98 of the water vole are high, the gestation period lasts 21 to 22 days, with four to seven embryos
99 per litter, 3 litters per year, and sexual maturity is reached at about five weeks.

100 2.3. Monthly physiological monitoring

101 From February 2019 through to June 2020, 13 successive rounds of captures were carried out
102 to perform monthly physiological monitoring (Table 1). Lack of data for some months is due
103 to winter climatic conditions, which made animal trapping unfeasible. Captures were
104 performed during the day on permanent meadows around Angle-bas (45° 42' 47" N, 2° 46'
105 12" E, alt. 887 m) in the French department of Puy-de-Dôme. Female fossorial water voles
106 (*Arvicola terrestris*) were caught with traps (Topcat®, Andermatt, France) placed into their
107 galleries. Traps were checked regularly (~every 15min) and sampling was performed right
108 away. For each animal, we recorded body weight and size (head and body, without tail) and
109 performed visual inspection of vaginal opening to assess sexual maturity. The number of
110 embryos was counted for each pregnant female, and non-pregnant lactating females were
111 identified by their developed nipples. Uterus and ovaries were collected and fixed in Bouin's
112 solution (Sigma, France) for 48h before being weighed and stored in 70% ethanol. The lateral
113 scent glands were frozen in dry ice and stored at -80°C.

114 For the analyses of monthly variations, only data from non-pregnant females were used. The
115 weights of the uterus, ovaries, and lateral scent glands were related to the body weight for
116 each animal, while the surface of lateral scent glands was related to the body size.
117 Uncorrected values are available in supplementary data (Sup.Table 1).

118 Monthly weather data (sunshine, rainfall, and average temperature) corresponding to the
119 monitoring period were obtained from the website www.infoclimat.fr.

120 2.4. Tissue collection for immunohistochemistry

121 Non-pregnant females caught in summer (July and August), and winter (November) (Figure
122 1A) were anaesthetised with isoflurane vapours, then euthanised by injecting an overdose of
123 pentobarbital and finally intracardially perfused with a sodium nitrite solution followed by 4%
124 paraformaldehyde (PFA) in phosphate buffer. Brains, ovaries, uterus, and lateral scent glands

125 were collected and postfixed in 4% PFA for 48h then weighed before being cryoprotected in
126 30% sucrose. Pituitaries were collected in NaCl buffer before being weighed.

127 2.5. Immunohistochemistry

128 Before immunohistochemical labelling, brains were frozen at -18°C, sliced in coronal free-
129 floating sections of 30µm, and stored in cryoprotective solution until analysed. Then, brain
130 sections were washed three times for 5 min in Tris buffer saline (TBS). Sections were
131 incubated for 30 min with 0.3% H₂O₂ in TBS to inhibit endogenous peroxidase activity and
132 for 2h in TBS containing 0.1% triton X100 with 2% normal donkey serum (NDS) to block
133 non-specific binding. Sections were then incubated for 72h at 4°C in TBST-NDS with a rabbit
134 anti-kisspeptin antibody (1:50000, AC564, (Franceschini et al., 2006), Table 2). After three 5
135 min washes, sections were incubated for 2h at room temperature (RT) in TSBT-NDS with a
136 donkey anti-rabbit antibody (1:1000, Jackson ImmunoResearch). Sections were washed three
137 times for 5 min and incubated for 1h at RT with an avidin/biotin system for signal
138 amplification (VECTASTAIN® Elite® ABC HRP Kit, PK-6100). Sections were then rinsed
139 three times for 5min in Tris-HCL and revealed with DAB 0.15 mg.mL⁻¹ dissolved in Tris-
140 HCL containing 0.01% H₂O₂ for 15 min. Finally, sections were mounted in TBS, dehydrated,
141 and coverslipped in DPX. The specificity of immunolabeling was checked on consecutive
142 sections of the arcuate nucleus by pre-adsorption for 24h of the primary antibody with the
143 kisspeptin 10 peptide (GeneCust, France) (Sup. figure 1).

144 2.6. Histological staining

145 One horn of each uterus was sectioned in its middle portion for sampling. Uterine horn
146 samples and ovaries were rinsed in phosphate buffer saline (PBS) for 24h. Then, they were
147 dehydrated in successive baths of 50%, 70%, and 90% ethanol, followed by three baths of
148 absolute ethanol for 30 min each. After one night at 4°C and 2h at RT in butanol, samples
149 were impregnated in paraffin two times at 60°C before final embedding. Once dried, samples
150 were sliced into 5µm sections and arranged on slides. Before staining, sections were
151 deparaffined in xylene two times for 5 min. Then, sections were rehydrated with successive
152 baths of absolute, 90%, 70% ethanol, and a final bath of tap water, two times for 5 min each.
153 Sections were then stained in hematoxylin for 30 sec and cleared under tap water. After a
154 quick bath in 70% ethanol, sections were stained again in eosin for 5 sec. Finally, sections
155 were dehydrated and coverslipped in DPX.

156 Lateral scent glands were embedded in OCT (Tissue-Tek), frozen in dry ice, and sliced into
157 10 µm sections using a cryostat. Then, sections were dried and stained with the same protocol
158 as described above.

159 2.7. RNA extraction, Reverse Transcription-PCR and Cloning

160 The complete open reading frame for *Kiss1* was obtained using hypothalamic mRNA from a
161 single male water vole. All procedures have been described in detail elsewhere (Lomet et al.,
162 2018). RNA was extracted using TriReagent (Sigma). Concentration and purity of the sample
163 were determined with Nanodrop 2000 (ThermoScientific), and integrity was checked by
164 standard agarose gel electrophoresis. cDNA was synthesised using Omniscript RT kit
165 (Qiagen) and Oligo-dT primers (Eurofins, Germany). cDNA was cloned using a standard
166 homology procedure whereby multiple rodent *Kiss1* sequences were aligned to guide PCR
167 primer design (see Table 2). PCR was performed using AccuPrime GC-rich DNA polymerase
168 (from Thermofisher). PCR reactions were loaded on a 1.5% agarose gel and migrated at 80-
169 110V in Tris-EDTA-Acetic acid (TEA) buffer for ~30min. PCR fragments were extracted on
170 gel and cloned in pBS (Promega). Six independent clones were sequenced (Eurofins).

171

172 2.8. Image acquisitions and quantifications

173 Images of sections of brains, uterine horns, ovaries, and lateral scent glands were acquired
174 with AxioScan.Z1 slide scanner (Carl Zeiss France).

175 The measure of the uterine horn surface was performed on two sections separated by 100 µm
176 with the combination of the surface of myometrium and endometrium without the lumen. To
177 evaluate the existence of a period of anoestrus, which would be characterized by anovulation
178 and therefore by the absence of corpora lutea, the number of *corpora lutea* was counted on
179 three sections distributed over the entire surface of one ovary for each animal. Haemorrhagic
180 and mature *corpora lutea* with well-defined luteinised and non-necrotic theca and granulosa
181 cells were defined as being “recent”. The two ovaries were inspected to determine the
182 percentage of females with recent *corpora lutea* in summer (July + August, n=11) and in
183 winter (November + January, n=9).

184 Quantifications of kisspeptin-ir fibre density were performed on three sections at the anterior,
185 median, and caudal levels of the arcuate nucleus (corresponding to the plates 43, 48, and 51 of
186 the Paxinos and Franklin mouse brain atlas). The counting surface was 0.029 mm² on each
187 side of the third ventricle. Images were analysed with ImageJ software (NIH, ver.1.52p).

188 Images were binarised, background was subtracted, and the area covered by the fibres was
189 measured after thresholding. No difference was detected between the three levels of arcuate
190 nucleus. So, statistical analysis was performed on the average of the quantifications of these
191 three levels to have a global overview of the arcuate nucleus.

192 Only few kisspeptin-ir fibres and cell bodies were detected in the AVPV (anteroventral
193 periventricular nucleus) which prevented us from performing any relevant quantification.

194 2.9. Statistical analysis

195 Results are shown as Tukey's boxplot with each individual (including outliers) represented by
196 a circle; means are indicated by a cross. Normality of data distribution was verified with the
197 Shapiro-Wilk test. According to the normality, data were analysed either by one-way
198 ANOVA followed by Tukey's multiple comparisons test or by Kruskal-Wallis followed by
199 Dunn's test. To simplify interpretation of the data related to the annual monitoring, only
200 posthoc comparisons with the month of January are presented. All other pairwise comparisons
201 are available in supplementary data (Sup. Table 2). The effect of season on the weight of
202 pituitary and the density of kisspeptin-ir fibres were analysed by unpaired Student's t-test.
203 GraphPad Prism 8.4.3 (GraphPad Software Inc, San Diego, California USA) was used to
204 perform statistical analyses and to draw the graphs.

205 3. Results

206 The average body weight of non-pregnant females significantly differs throughout the year
207 (Table 1, one-way ANOVA, $p=0.0013$). The maximum average body weight was observed
208 during spring, from April through June, while the minimum was observed during winter, in
209 January (98g vs 75g, $p=0.0028$). The average body size also differed throughout the year
210 (Table 1, Kruskal-Wallis, $p=0.0053$) even though no pairwise comparison reached statistical
211 significance.

212

213 Pregnant females were observed from the end of February through October 2019, then again
214 from April 2020 onwards (Figure 1A). The maximum percentage of pregnant females (58%
215 of sexually mature females) was observed in June 2019. During this month, among the non-
216 pregnant females, 28% were lactating. Despite the presence of sexually mature females, a lack
217 of pregnant females was observed between November 2019 and the end of February 2020.

218 The average number of embryos per female (Figure 1B) was close to 4, but varied from 1 to
219 7, and did not display statistically significant variation.

220

221 The weight of the ovaries displayed a significant annual rhythm (Figure 2A, one-way
222 ANOVA, $p < 0.001$). The heaviest ovaries were observed at the end of April 2019, while the
223 lightest ovaries were found in January 2020 (0.0219%.BW vs 0.0018%.BW, $p < 0.001$). In the
224 ovaries, the number of *corpora lutea* was quantified, and a significant difference was detected
225 (Figure 2B&C, Kruskal-Wallis, $P < 0.001$) with a maximum number of *corpora lutea* at the
226 end of April 2019 and a minimum in January 2020 (3.16 vs 0.25, $p = 0.0350$). Recent *corpora*
227 *lutea* were found only in females trapped in summer (July/August) compared to winter
228 (November/January) (8/11 vs 0/9, Fisher's exact test, $p = 0.0014$, Figure 2C).

229

230 Consistent with data for body and ovarian weight, the uterine weight showed a significant
231 monthly variation (Figure 2E, Kruskal-Wallis, $p < 0.001$). These variations followed a
232 sinusoidal-like profile with a progressive increase, up until the maximum was reached in June
233 2019, then followed by a large 10-fold decrease until minimum weight was attained in
234 January 2020 (0.1988% of BW, 0.0186% of BW, $p < 0.001$). Then, from January through to
235 June 2020, the uterine weight increased again. As for the uterine weight, a significant monthly
236 variation of the surface of uterine horn sections was detected (Figure 2F and G, one-way
237 ANOVA, $p < 0.001$). The surface of the myometrium and endometrium was significantly
238 larger in June compared to January (2.06 vs 0.27 mm², $p = 0.0027$).

239

240 Histological observations of sections after staining of the lateral scent glands showed that
241 numerous sebaceous glands are located under the epidermis. The lipidic secretions are
242 produced by the vacuolated cells contained inside alveoli of different lobules (Figure 3A&B).
243 These lateral scent glands showed a significant monthly variation of both their size and
244 weight (Figure 3C&D, Kruskal-Wallis, $p < 0.001$ for both). Similar to the uterus and ovaries,
245 variations of lateral scent glands size and weight followed a sinusoidal-like profile with
246 maximal development in September 2019, followed by regression until a nadir was reached in
247 January 2020 ($p < 0.001$ for both).

248

249 The weight of the pituitary was significantly higher in voles trapped in summer (July and
250 August) compared to the ones trapped in winter (November) (4.08mg vs 2.83mg, Student t-
251 test, $p = 0.0118$; Figure 4C). In the arcuate nucleus of the hypothalamus, a significantly lower

252 density of immunoreactive kisspeptin fibres was detected in voles trapped in summer
253 compared to the ones trapped in winter (Student-t-test, $p < 0.001$; Figure 4A&B).

254

255 The complete open reading frame for *Kiss1* was obtained after cloning and sequencing by
256 using hypothalamic mRNA from a single male European water vole (*Arvicola terrestris*). The
257 cDNA for *Kiss1* has been deposited in Genbank under accession number MT922570. The
258 *Kiss1* cDNA is predicted to yield a precursor protein of 136 amino acids, which shows very
259 high similarity with KISS1 sequences of multiple other rodents (Figure 4D). In particular, the
260 sequence of the water vole KP10 peptide (last 10 amino acids at the C-term of the mature
261 peptide) is identical to that of all other rodents. Akin to what has been found for other
262 mammals, the European water vole *Kiss1* open reading frame exhibits a high 66% GC content
263 (mouse 67%, human 67%, sheep 74%, horse 75%).

264 **4. Discussion**

265 Our extensive field study, relying on monthly physiological monitoring performed over a
266 period of ~1.5 years uncovers dramatic seasonal rhythmicity in multiple morphological and
267 neuroendocrine markers along the hypothalamo-pituitary-gonadal axis of female fossorial
268 water voles. These features indicate that *Arvicola terrestris* displays marked seasonal
269 breeding, akin to what is found in other long-day breeders.

270 In our female fossorial water voles, a change in the body weight was observed throughout this
271 monitoring with heavier and lighter animals observed during spring/summer and winter,
272 respectively, which was previously described in other rodents known as seasonal breeders
273 (Jones et al., 2020; McKeever, 1963; Wade and Bartness, 1984).
274 This change in body weight can also be linked to the renewal of the population. In the wild,
275 even though a study reported that some water voles may live up to 2 years, the average
276 lifespan is 4-5 months (Stoddart, 1971). In another study, the lifespan was estimated to be
277 between 2 and 2.5 years, with a marked decrease in the number of older animals in autumn
278 (Ventura et al., 1991). No difference in body size of animals was detected between winter and
279 any other season. The vaginal opening inspection associated with the measure of body size
280 confirmed the sexually mature state of females.

281

282 Pregnant and lactating females were observed between March and October. While sexually
283 mature females were occasionally found also for the remainder of the year (from November to

284 February), none of these were identified as being pregnant. This is fully consistent with earlier
285 findings, which defined the breeding period of water voles as being discontinuous throughout
286 the year (Airoldi, 1978). Pregnant females were mainly observed from March through to
287 October, even though some could also be observed during mild winters (Meylan and Airoldi,
288 1975). Our current observations strengthen the existence of a consolidated period of sexual
289 rest during winter in female water voles.

290 We then assessed whether seasonal breeding rhythmicity could be correlated with rhythmic
291 anatomical and neuroendocrine variations at all levels of the hypothalamo-pituitary-gonadal
292 axis. An increase in the weight of the ovaries was observed between late winter and early
293 summer, followed by a progressive regression until winter as previously described in Belding
294 ground squirrel and Pine voles (McKeever, 1963; Valentine and Kirkpatrick, 1970). This
295 increase in ovarian weight was associated with a higher number of *corpora lutea* in spring and
296 summer, thus indicating a higher rate of ovulation than in winter, as reported in bank and field
297 voles (Brambell and Rowlands, 1936; Thibault et al., 1966). In addition, no recent *corpora*
298 *lutea* were found in winter, which may suggest a possible winter anoestrus in some female
299 water voles.

300 A seasonal change in uterine weight was also observed. Female water voles trapped in spring
301 and early summer presented the heaviest uterus with myometrium and endometrium
302 considerably more developed than in winter. Uterine tissues are known to be highly sensitive
303 to oestradiol (Brody and Wiquvist, 1961; Galand et al., 1971), and the increase of uterine
304 weight observed in spring and early summer suggests a higher level of estradiol production by
305 ovaries during this period.

306 In parallel to the reproductive organs, seasonal changes of lateral scent glands were identified
307 with maximal development in summer and a dramatic regression in winter, as described
308 earlier in male water vole (Stoddart, 1980). These sebaceous glands were shown to be
309 androgen-dependent in the male water vole (Stoddart, 1972), as in male and female hamsters
310 and gerbil (Frost et al., 1973; Lucky et al., 1986; Thiessen et al., 1971; Vandenberg, 1973).
311 Indeed, the development of these glands was used as an external marker of gonadal activity in
312 gerbil (Thiessen et al., 1968). In water vole, it was further proposed that the scent glands are
313 involved in the attraction of the sexual partner (Stoddart et al., 1975). In this context, we
314 recently identified season-specific signatures of the volatile olfactory compounds produced by
315 the lateral scent glands (Nagnan-Le Meillour et al., 2019).

316 Moving backwards along the hypothalamo-pituitary-gonadal axis, we found that the pituitary
317 is heavier in animals trapped in summer, compared to those trapped in winter. Similar
318 observations were made in females field voles and Belding ground squirrel (Al-Khateeb and
319 Johnson, 1971; McKeever, 1963). Furthermore, male field voles also show increased pituitary
320 weight during the breeding season, and this is associated with increased levels of LH and
321 FSH, both in the pituitary and in the plasma (Craven and Clarke, 1986). The increased level of
322 gonadotropins observed in female field and prairie voles during the breeding period is
323 consistently correlated with higher ovulation rates (Martinet and Meunier, 1975; Moffatt et
324 al., 1995). The heavier pituitary weight in summer may also be due to an increased number of
325 lactotroph cells, which produce prolactin to stimulate milk production in nursing females
326 (Carretero et al., 2003).

327 Finally, we investigated potential seasonal regulation at the central hypothalamic level, by
328 assessing levels of kisspeptin peptide, the most potent secretagogue of GnRH. We first cloned
329 the *Kiss1* cDNA in this species. The deduced sequence of the water vole KISS1 precursor
330 protein is highly similar to the sequence of other rodents. Moreover, the water vole KP10
331 sequence is identical to the mouse KP10 sequence against which the AC564 antibody is
332 directed. Using this KP10-specific antibody, we showed that the density of kisspeptin-ir fibres
333 in the hypothalamic arcuate nucleus is much lower in female voles trapped in summer than in
334 those trapped during winter. In seasonal species, kisspeptin levels in the arcuate nucleus are
335 often correlated to the reproductive state, with more neurons and fibers observed during the
336 breeding season (summer for rodents, and winter for sheep of temperate regions of the world),
337 compared to the non-breeding season. This is consistent with the role of kisspeptin as a major
338 GnRH secretagogue (Messenger et al., 2005). Our findings in female water voles may therefore
339 appear counterintuitive, in that levels of kisspeptin-ir are opposite to this general rule.
340 However, similar results have been observed in Siberian (*Phodopus sungorus*) and European
341 hamsters (*Cricetus cricetus*), in which a low density of kisspeptin-ir is seen in animals
342 maintained under long photoperiod (summer condition) compared to those maintained under
343 short photoperiod (winter) (Greives et al., 2007; Rasri-Klosen et al., 2017; Sáenz De Miera et
344 al., 2014). This result could be due to the potent negative feedback exerted by sex hormones,
345 particularly estradiol, onto arcuate kisspeptin neurons during the reproductive period (Gottsch
346 et al., 2009; Smith et al., 2005), which vanishes during the period of sexual rest. The highest
347 density of kisspeptin fibres in winter might also result from diminished secretion, hence the

348 accumulation of the peptide, which is actively secreted during the breeding period (Rasri-
349 Klosen et al., 2017).

350 Altogether, our results considerably substantiate the hypothesis that female water voles
351 display a marked annual cycle of reproduction, with breeding during spring/summer and a
352 sexual rest period during autumn/winter. Our data provide evidence for these seasonal
353 regulations at all levels along the hypothalamo-pituitary-gonadal axis (Figure 5). In most
354 seasonally breeding mammals living at temperate latitudes, daylength is the main proximate
355 driver of seasonal breeding, as demonstrated in the field vole (Baker and Ranson, 1933,
356 1932a, 1932b), through mechanisms that involve the neurohormone melatonin produced and
357 secreted by the pineal gland (for review see Dardente *et al.*, 2019). However, it is noteworthy
358 that additional cues, among which social interactions, stress and food availability, are able to
359 modulate the impact of photoperiod to some extent (Dardente et al., 2016; Delgadillo et al.,
360 2020). Voles are no exception to this general statement. Indeed, the density of vole population
361 and quality of the feed resource can impact fertility of female voles in the field (Martinet et
362 al., 1969; Pinot et al., 2014). Moreover, mild temperature associated with better resource
363 availability seems to explain the continuous reproduction along the year of the fossorial water
364 voles located in Northwest Spain (Somoano et al., 2016).

365 In summary, our data demonstrate that female water voles are seasonal breeders. Regulations
366 of the reproductive axis throughout the year are responsible for the alternation between
367 periods of breeding and sexual rest. While photoperiod is the main environmental factor
368 governing seasonal changes in mammals, its direct involvement in seasonal breeding of the
369 water vole remains to be demonstrated rigorously. Finally, the relative impact of photoperiod
370 and additional cues such as food availability and temperature remains to be determined in
371 both female and male water voles.

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558

559 **Figure captions:**

560 **Table 1.** Summary for each capture of the number of adult females not pregnant, the number
561 of samples used for staining and immunohistochemistry, and the average body weight and
562 height. Only significant posthoc comparisons to January are indicated on graphs (* $p<0.05$. **
563 $p<0.01$).

564 **Table 2.** Characteristics of antibodies and primers used for Immunohistochemistry and PCR.

565 **Figure 1. Female voles caught in the field display a marked annual rhythmicity in**
566 **pregnancy.** (A) Percentage of pregnant, non-pregnant lactating and non-pregnant females
567 observed throughout the monitoring. (B) The average number of embryos per female. (Results
568 are shown as Tukey's boxplot with each female represented by circles and means indicated by
569 a cross).

570 **Figure 2. Female voles caught in the field display a marked annual rhythmicity in their**
571 **reproductive organs.** Graphs show monthly variations in (A) ovarian weight. (B) *corpora*
572 *lutea* number. (E) uterine weight and (F) uterine horn section. Only significant posthoc
573 comparisons to January are indicated on graphs (Results are shown as Tukey's boxplot with
574 outliers represented by circles and means indicated by a cross. * $p<0.05$. ** $p<0.01$.
575 *** $p<0.001$). (C) Percentage of females with recent *corpora lutea* in summer (July+August,
576 $n=11$) or in winter (November+January, $n=9$). (D&G) Histological staining of ovaries and
577 uterine horn sections to illustrate the difference between July and January. (e: endometrium;
578 m: myometrium; CL: *corpora lutea*).

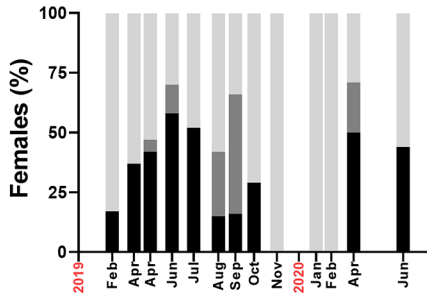
579 **Figure 3. Female voles caught in the field display a marked annual rhythmicity in their**
580 **lateral scent glands.** (A) Photography indicates the position and details the appearance of the
581 lateral scent gland. (B) Images of hematoxylin and eosin stains of sections of lateral scent
582 glands. The double arrow indicates the thickness of the sebaceous gland layer. (SG: sebaceous
583 gland; e: epidermis). (C&D) Monthly changes in the size and weight of lateral scent glands of
584 female water voles. Only significant posthoc comparisons to January are indicated on graphs

585 (Results are shown as Tukey's boxplot with outliers represented by circles and means
586 indicated by a cross. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$).

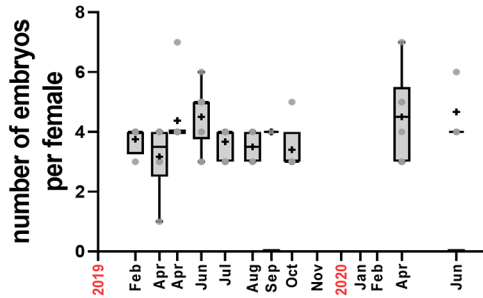
587 **Figure 4. Female voles caught in the field display a seasonal change in the level of**
588 **kisspeptin and pituitary weight.** (A&B) Representative images and graph of
589 immunoreactive kisspeptin fibres density in the arcuate nucleus of summer-trapped and
590 winter-trapped females (n=7 for both). Red squares indicate the counting surface. (ARC:
591 arcuate nucleus, 3V: Third ventricle, ME: median eminence). (C) Pituitary weight of female
592 water voles trapped in summer (n=8) or in winter (n=7) (Results are shown as Tukey's
593 boxplot and means indicated by a cross. * $p < 0.05$, *** $p < 0.001$). (D) Alignment of kisspeptin
594 sequence in *Arvicola terrestris* and other selected rodents (*Microtus ochrogaster*,
595 *Mesocricetus auratus*, *Cricetus griseus*, *Peromyscus leucopus*, *Meriones unguiculatus*,
596 *Arvicanthis niloticus*, *Rattus norvegicus* and *Mus musculus*). Yellow: positions unique to
597 *Arvicola terrestris*; Bold Green: Kp10; Bold Red: dibasic cleavage site + amidation (based on
598 mouse Kp52 sequence). Asterisks indicate positions which have a single fully conserved
599 residue. Colons indicate conservation between amino acid groups of strongly similar
600 properties. Periods indicate conservation between amino acid groups of weakly similar
601 properties.

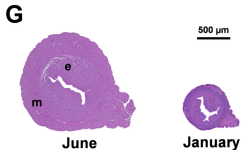
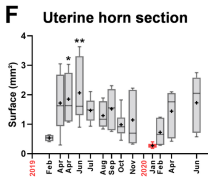
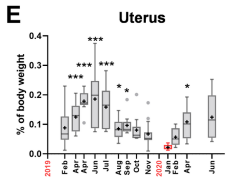
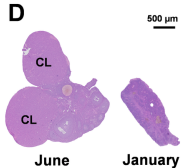
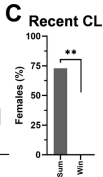
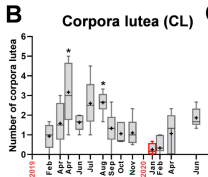
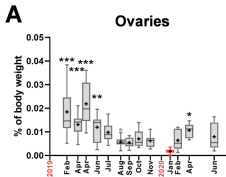
602 **Figure 5. The alternation of the breeding and sexual rest periods correlates with the**
603 **annual rhythmicity in regulations of the hypothalamo-pituitary-gonadal axis;** and in
604 parallel with the indication of the day length, the monthly average temperature, sunshine, and
605 rainfall throughout the monitoring (infoclimat.fr). Hypothesis: the lowest kisspeptin fibres
606 immunoreactivity in arcuate nucleus during the breeding season compared to the non-
607 breeding season might be due to enhanced secretion of kisspeptin (i.e. less storage) along with
608 strong negative feedback of estradiol onto the arcuate kisspeptin neurons, thereby mitigating
609 *Kiss1* at the transcriptional level. In this scenario, accumulation of kisspeptin in winter would
610 reflect reduced kisspeptin secretion (i.e. storage) along with reduced estradiol feedback. (1)
611 (Sophie Messager et al., 2005); (2) (Belchetz et al., 1978); (3) (Brody and Wiqvist, 1961;
612 Galand et al., 1971; Greep et al., 1942; Stoddart, 1972). (GnRH, Gonadotropin release
613 hormone; LH, Luteinizing hormone; FSH, Follicle-stimulating hormone; E2, Estradiol; P4,
614 Progesterone).

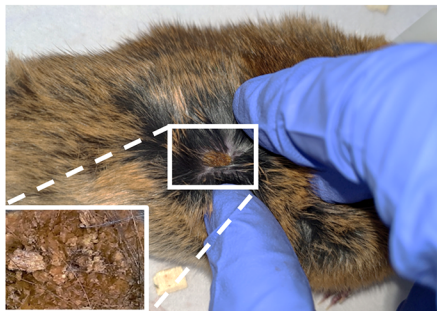
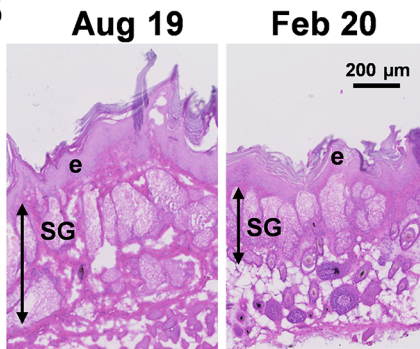
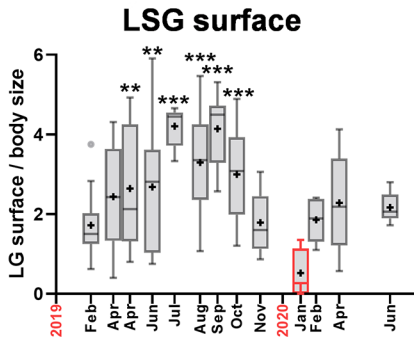
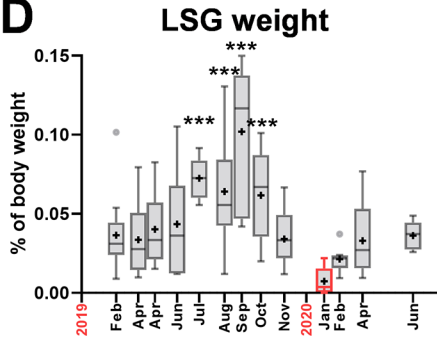
A ■ Pregnants ■ Lactating ■ Non-pregnants

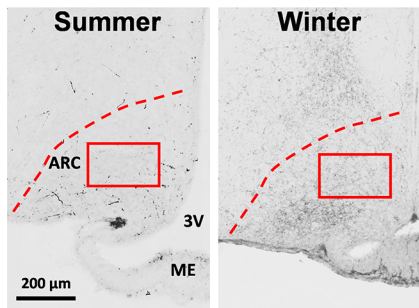


B



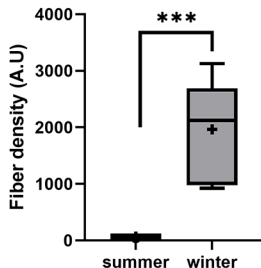
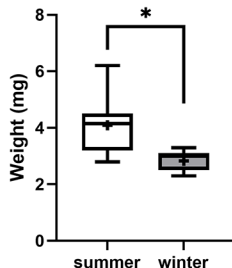


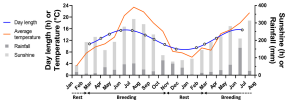
A**B****C****D**

A**D**

Micr_ochr	MTALASWQLLLLLLCITTFGEP-----LANPGPTGQQSG-SQELINAWKKGPRCAEGKSGVTGLRAR--RTSPCPPVDS
Arvi_terr	MTALASWQLLLLLLCITTFGEPLAKVAPLANPGPTGQQSG-SQELINVWEKGPRCAEGKSGVAGLRAR TRRTL LPCPPVES
Meso_aura	MTTLASWQLLLLLLCVATFGEPLAKVAPVNPNGPTGQRP-GPQELINAWKEGPRCAQKPKGIAGLRAR--RTSPCPPVES
Cric_gris	MTALASWQLLLLLLCVTFIFGEPLAKVAPVNPNGPTGQRP-GPQELINAWKEGPRCAERKPGVAGLRAR--RTSPCPPVES
Pero_leuc	MTSLASWQLLLLLLCVATFGEPE---PAKVAPGPTGQRP-GPQELINAWKEGPRCAERRPGVAGLRAR RTS PPCPPVES
Meri_ungu	MPPLASWQLLLLLLFVTTTFGEPLAKVAPVVKPGPSGRRRPGLQELVNAWEKGPRCAERKPGAAGPRAR--RASPCPPVES
Arvi_nilo	MISLASWQLLLLLLCVATFGERLAKVAPVNPNGHTGQWSG-SQELVNAWEKSPRYAESKPEAAGLRAR--RTSPCLPVES
Ratt_norv	MISLASWQLLLLLLCVASFGEPLAKMAPVNPPEPTGQQSG-PQELVNAWQKGPARYAESKPGAAGLRAR--RTSPCPVVEN
Mus_mus	MISMASWQLLLLLLCVATYGEPLAKVAPLVKPGSTGQQSG-PQELVNAWEKESRYAESKPGSAGLRAR--RSSPCPFVEG
	* .:***** :::** . * :*:.* ***:**:* * *: .:.* *** . ** **:

Micr_ochr	SAGRQRPACTAR-GRLIPTPRGAVLVQREKDL SAYNWN SFGLRY GRR QATRAARAARG--
Arvi_terr	SAGRQRPACAA L -GRLIPTPRGAVLVQREKDL SAYNWN SFGLRY GRR QAARAARAARG--
Meso_aura	SAGRLRPVCSAR-SRLIPAPRGAVLVQREKDL SAYNWN SFGLRY GRR ARAARAATPARG--
Cric_gris	SAGRPRPVCAAR-SRLIPAPRGAVLVQREKDL SAYNWN SFGLRY GRR QAARAARAATRAARG
Pero_leuc	SSGRQRPCTAAGSRLIPAPRGAVLVQREKDL SAYNWN SFGLRY GRR QAARAARG-----
Meri_ungu	SSGRQRP LCAAG-SRLIPAPRGAVLVQREKDL SAYNWN SFGLRY GRR QAARAAPAARG---
Arvi_nilo	SAGHQRP LCAAR-SRLIPAPRGSVLVQREKDL SAYNWN SFGLRY GRR QAARAQAQ---
Ratt_norv	PTGHQRP PCATR-SRLIPAPRGSVLVQREKDL SAYNWN SFGLRY GRR QVARAARG-----
Mus_mus	PAGRQRP LCAASR-SRLIPAPRGAVLVQREKDL ST YNWNS FGLRY GRR QAARAARG-----
	.:*: ** *:: .****:***:*****:*:***** . *

B**Kisspeptin fibres****C****Pituitary**



GnRH (1)	Kisspeptin in ARC immunoreactivity		-	+
		Pituitary	Weight	+
LH, FSH (2)	Ovaries	Weight	+	+
		number of corpora lutea	+	+
		Recent Corpora lutea	+	-
E2, P4 (3)	Uterus	Weight	+	+
		Surface	+	+
	Lateral Scent Glands	Weight	+	+
		Surface	+	+

Date of capture			Nb of voles	Staining Ovaries - Uterus	IHC & Pituitary	Body weight (g) ± SEM	Body size (cm) ± SEM	
2019	February	26	19	6-6		85.8 ± 2.0	14,5 ± 0,1	
	April	03	10	6-6		92.9 * ± 4.2	14,5 ± 0,3	
	April	30	11	5-6		95.9 ** ± 3.3	14,5 ± 0,3	
	June	05	7	5-6		98.0 ** ± 5.3	16,0 ± 0,3	
	July	11	8	5-5	3-4	95.3 * ± 4.1	15,0 ± 0,4	
	August	21	23	6-6	4	86.1 ± 2.1	14,5 ± 0,2	
	September	18	10	6-6		87.3 ± 2.7	14,5 ± 0,3	
	October	19	12	5-5		90.3 ± 2.9	15,5 ± 0,2	
	November	26	15	5-5	7	89.3 ± 2.2	15,0 ± 0,2	
	2020	January	30	9	4-6		75.0 ± 3.1	15,0 ± 0,3
		February	24	8	5-6		82.6 ± 3.5	15,0 ± 0,4
April		01	7	5-6		96.7 * ± 6.4	15,0 ± 0,3	
June		22	5	5-5		89.8 ± 6.5	15,5 ± 0,3	

Antibodies	Catalog # and RRID	Dilution
Rabbit anti-kisspeptin	AC564 ; AB_2296529 Home	1:50000
Biotin SP Donkey anti-rabbit	711-065-152 Jackson ImmunoResearch	1:1000

PCR Primers	5' → 3' sequence
Forward	GAACTGCCTCTTCTGCCAG
Reverse	ACTGGGCTCGCAATCCACC