

1 **Sex recognition by odour and variation in the uropygial gland secretion in**
2 **starlings**

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18 Running headline: Odour-based sex recognition in a bird

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21 1. Although a growing body of evidence supports that olfaction based on chemical
22 compounds emitted by birds may play a role in individual recognition, the possible role
23 of chemical cues in sexual selection of birds has been only preliminarily studied.

24 2. We investigated for the first time whether a passerine bird, the spotless starling
25 *Sturnus unicolor*, was able to discriminate the sex of conspecifics by using olfactory
26 cues and whether the size and secretion composition of the uropygial gland convey
27 information on sex, age and reproductive status in this species.

28 3. We performed a blind choice experiment during mating and we found that starlings
29 were able to discriminate the sex of conspecifics by using chemical cues alone. Both
30 male and female starlings preferred male scents. Furthermore, the analysis of the
31 chemical composition of the uropygial gland secretion by using gas chromatography–
32 mass spectrometry (GC-MS) revealed differences between sexes, ages and reproductive
33 status.

34 4. In conclusion, our study reveals for first time that a passerine species can
35 discriminate the sex of conspecifics by relying on chemical cues, and suggests that the
36 uropygial gland secretion may potentially function as a chemical signal used in mate
37 choice and/or intra-sexual competition in this species.

38

39 **Key-words:** Avian olfaction, Chemical ecology, *Sturnus unicolor*, Sex-recognition,
40 Uropygial gland

41

42 **Introduction**

43 Hitherto birds have been widely regarded as relying primarily on visual and auditory
44 stimulus during communication. By contrast, far less is known about the role of
45 chemical communication in birds. This may reflect the general belief that birds have a
46 poor sense of olfaction, although a growing body of novel evidence suggests that birds
47 have an olfactory apparatus similar in structure and function to that of other vertebrates,
48 and that they can use odours in several biologically relevant contexts (for reviews see
49 Hagelin & Jones 2007; Balthazart & Taziaux 2009; Caro & Balthazart 2010). For
50 example, it has been shown that birds may use the sense of smell to discriminate
51 aromatic plants (Petit *et al.* 2002; Gwinner & Berger 2008). Olfaction may also function
52 in orientation and navigation (Wallraff 2004; Nevitt & Bonadonna 2005), in prey
53 detection (Nevitt, Veit & Kareiva 1995; Cunningham, Castro & Potter 2009) and it may
54 also help to assess predation risk (Amo *et al.* 2008; Roth, Cox & Lima 2008; Amo,
55 Visser & van Oers 2011).

56 At the intra-specific level, evidence suggests that olfaction based on chemical
57 compounds emitted by birds may also play a key role in individual recognition (Caro &
58 Balthazart 2010). For example, birds have been shown to recognize their own nest on
59 the base of chemical cues (e.g. Bonadonna *et al.* 2004; Caspers & Krause 2011).
60 Procellariiformes are able to discriminate the scent of their partners from the scent of
61 other conspecifics (Bonadonna & Nevitt 2004; Jouventin, Mouret & Bonadonna 2007).
62 In ducks, olfaction may play a role in courtship behaviour, as male domestic ducks *Anas*
63 *platyrhynchos* with the olfactory nerve sectioned exhibited a significantly inhibited
64 sexual behavior (Balthazart & Schoffeniels 1979). Also, in crested auklets *Aethia*
65 *crisatella*, it has been shown that chemical cues may play a role in their social
66 behaviour (Hagelin 2007a). Finally, Hirao and collaborators (2009) have found that in

67 domestic chickens *Gallus gallus*, mate preference involves olfaction in males and that
68 the female's uropygial gland acts as a source of social odour.

69 Surprisingly, although evidence suggests a role for olfaction in individual
70 recognition, the possible role of chemical signals in sexual selection has been
71 comparatively far less studied in birds than in other taxa (Hagelin 2007b). For example,
72 at an intra-specific level, mammal scents have been shown to vary between individuals
73 and to reveal body condition, parasite load, health state and even genetic compatibility
74 (e.g. Major Histocompatibility Complex, Brennan & Keverne 2004). Therefore, odours
75 can be used in intrasexual interactions to assess the dominance status of rivals (e.g.
76 Arakawa *et al.* 2008) and/or to select potential partners (Johansson & Jones 2007;
77 Thomas 2011). However, it still remains unknown whether the scent that a bird releases
78 can provide valuable information about aspects of individual quality that may be useful
79 during competition for mates and mate choice.

80 A logical first step to determine the possible role of chemical cues in sexual
81 selection in birds is to analyse whether birds are able to discriminate the sex of
82 conspecifics by using chemical cues. To our knowledge, only two previous studies have
83 aimed to do so finding contrasting results. In a first study, Bonadonna *et al.* (2009)
84 failed to demonstrate odour sex recognition by conspecifics in the Antarctic prion
85 (*Pachyptila desolata*) during the incubation period, even when previous work had
86 demonstrated that individuals of this species could recognize their partners based on
87 olfaction (Bonadonna & Nevitt 2004). On the other hand, Zhang *et al.* (2010) found that
88 female budgerigars (*Melopsittacus undulatus*) were able to distinguish males from
89 females via body odour. More studies within this field in different bird orders
90 performed during the relevant mate choice period are clearly needed to disclose general
91 trends about the possible role of chemical signals in sexual selection of birds.

92 The uropygial gland secretion is considered as the main odour source in birds.
93 This secretion is a mixture of monoester and diester waxes, tryglicerides, fatty acids,
94 and hydrocarbons, although its composition varies widely among avian groups (Jacob &
95 Ziswiler 1982). It contains both volatile and non-volatile compounds in the form of
96 waxy fluids that birds collect and spread on their feathers during preening (Jacob &
97 Ziswiler 1982). Therefore, the chemical components of the uropygial secretion are also
98 present in the feathers of birds (Soini *et al.* 2007; Mardon, Saunders & Bonadonna
99 2011). The fact that the gland secretory activity as well as the chemical components of
100 uropygial secretions vary between seasons (e.g. Jacob *et al.* 1979; Reneerkens, Piersma
101 & Sinninghe Damsté 2002), sexes (e.g. Jacob *et al.* 1979; Piersma, Dekker & Sinninghe
102 Damsté 1999; Zhang, Sun & Zuo 2009; Mardon *et al.* 2010; Whittaker *et al.* 2010;
103 Zhang *et al.* 2010), age classes, diets (e.g. Sandilands *et al.* 2004a,b) and hormone
104 levels (e.g. Whelan *et al.* 2010) suggests that these secretions may provide important
105 information during intra-specific interactions, particularly in sex recognition and mate
106 choice.

107 We experimentally investigated for the first time whether a passerine bird, the
108 Spotless starling *Sturnus unicolor* L., can discriminate the sex of conspecifics by using
109 olfactory cues during the mating period. We also analysed sexual and seasonal variation
110 in the size of the uropygial gland as well as age, sexual and seasonal variation in the
111 composition of its secretion aiming to ascertain its potential as a chemical cue
112 functioning in sex recognition in this species. Spotless starlings offered an ideal model
113 to cope with our objectives as several studies have shown that a close relative species,
114 the European starling *Sturnus vulgaris* L., can detect chemical compounds in different
115 contexts (e.g. White & Blackwell 2003). Homing experiments have shown that starlings
116 use olfaction for orientation (Wallraff *et al.* 1995). Starlings also have the capability to

117 discriminate the scent of the aromatic plants they introduce in their nests (Clark &
118 Mason 1987). This capacity has an innate component although it may be supplemented
119 by learning (Gwinner & Berger 2008). Olfactory capacity also shows seasonal changes,
120 with starlings exhibiting an elevated responsiveness to odours during the breeding
121 season (Clark & Smeranski 1990; De Groof *et al.* 2010). All these evidences together
122 would suggest that chemical cues may play an important role in the reproductive period
123 of starlings, and therefore, that they may have an intraspecific signalling function.

124 For our purposes, during the mating period, we tested sex recognition by
125 conspecifics by offering the scent of a male and a female to experimental individuals in
126 an olfactometry chamber. We predicted that if birds were able to discriminate the sex of
127 conspecifics, they should choose the side of the chamber containing the scent of a
128 conspecific of the opposite sex. In addition, we analysed the chemical composition of
129 the uropygial gland secretion in relation to sex, age and reproductive period of birds by
130 using gas chromatography–mass spectrometry (GC-MS). We also measured the
131 uropygial gland size searching for differences between sexes and reproductive states in
132 the secretory activity of the gland on the knowledge that the size of the gland is
133 positively correlated with the quantity of produced secretion (Martín-Vivaldi *et al.*
134 2009). We predicted differences between sexes, ages, and reproductive periods in the
135 chemical composition of the uropygial gland secretion of starlings. We predicted that
136 females may have larger glands than males, and they may exhibit larger uropygial
137 glands during the rearing of nestlings than earlier in the reproduction, as has been
138 observed in other species (e.g. Martín-Vivaldi *et al.* 2009).

139

140 **Materials and methods**

141

142 STUDY SPECIES

143

144 The spotless starling is a medium-sized, hole-nesting passerine that frequently breeds in
145 colonies. Males compete for nest sites and try to attract females to them (Cramp 1998),
146 being thus the females who choose the males. Incubation, which takes around 14 days,
147 is done mainly by females, whereas parental care is provided by both members of the
148 pair (Cramp 1998). The nestling period lasts approximately 21-22 days (Cramp 1998).

149 We performed the experiment in March 2010, when starlings are pairing and
150 building nests, in a spotless starling population breeding in nest-boxes in Guadix
151 (37°18' N, 3°11' W), south-eastern Spain. During the winter and mating period, starlings
152 roost in nest boxes. We visited nest-boxes before the sunrise and blocked their entries.
153 We captured by hand 39 adult starlings (18 males and 21 females). Starlings were
154 measured and ringed, and introduced in individual clean cotton bags until they were
155 tested. As soon as the experiment finished they were released. We also captured 10
156 additional birds (4 males and 6 females) to measure the size of their uropygial glands to
157 the nearest 0.01 mm with a digital calliper. In starlings, the gland has two lobes and
158 only one opening to the outside through a nipple structure. Three measurements were
159 taken: the maximum width, maximum length and 'height'. Width measures were taken
160 from the right lobe of the gland, while length was considered as the maximum distance
161 from the end of one lobe to the other. The 'height' of the gland was expressed as the
162 distance between the base of the lobes and the base of the nipple. These three
163 measurements were multiplied to obtain an estimate of the volume of the gland.
164 Although a rough approximation to real volume, this measure has successfully been
165 used to compare the size of the gland between sexes and reproductive periods in other
166 species (e.g. Martín-Vivaldi *et al.* 2009). We also took a sample of the uropygial gland

167 secretion of 9 of these birds (3 males and 6 females) by gently pressing the gland
168 against the border of the open of a 4 ml glass chromatographic vial. Vials were
169 maintained in cold conditions until collecting the secretions. In order to avoid
170 contamination, glass vials were previously autoclaved.

171 Later in the breeding season, we captured 89 different birds (76 females and 13
172 males) that were feeding their nestlings (5-8 days old) with a net trap inside the nest-
173 box. We weighed these birds with a spring balance (± 1 g) and measured their tarsus
174 length and uropygial gland with a calliper. We also took a sample of the uropygial gland
175 secretion from 23 birds (19 females and 4 males) following the above mentioned
176 protocol. Birds were released after ringing. Finally, we also extracted the uropygial
177 gland secretion from 15 12-14-day-old nestlings of 15 different broods selected at
178 random within our population.

179 Vials with the secretions were transported within the following 6 hours in a cool
180 box with cold-blocks in dark conditions to the lab, where they were stored in the dark at
181 - 20° C until analysed. Blank control vials were collected and processed in the same
182 way, and no compound was detected in their analyses.

183

184 BEHAVIOURAL STUDY

185

186 We performed sex-recognition experiments in an olfactometry chamber (see Fig. 1) in
187 indoor conditions. The device was composed by a small central plastic box (15 x 25 x
188 25 cm) where the experimental bird was introduced. It had a small 12 V PC fan that
189 extracted the air from the device creating a low-noise controlled airflow (Fig. 1). In each
190 test, a bird was introduced in the central box and maintained in the dark during 5
191 minutes. After that, a little lamp (6 V), was lighted in each one of the two choice

192 chambers connected to the central box, and the doors were opened. Each choice
193 chamber was divided into two sectors with screens. The farther sectors of the choice
194 chambers (15 x 25 x 25 cm) contained two little cages where donor birds of the
195 corresponding scent were situated. Both, the doors communicating the central chamber
196 with the choice chambers and the screens creating the sectors, were made with a dense
197 plastic mesh that allows air flow but avoids that birds could see through them. The
198 device was hermetically closed and was only opened at the farthest walls of the choice
199 chambers to allow air flow. The fan created two constant air flows, each one entering
200 across the openings located at the farthest walls of each choice chamber, passing
201 through the donor birds and crossing the central chamber, and going outside from the
202 device through the fan. Thus, the bird located in the central chamber received two
203 separate air flows, each one with the scent of the corresponding donor bird. Donor birds
204 were in darkness and in a reduced space, so they did not move or call. Therefore, the
205 experimental bird received the smell of the donor birds without watching or hearing
206 them. The room where the experiment was performed was in complete silence so the
207 experimenter could perceive any noise from any of the birds in the device. A similar
208 device has been used previously to successfully test bird preferences by different scents,
209 including conspecific scent, but with fresh feathers as scent donors (Hagelin, Jones &
210 Rasmussen 2003) instead of live birds.

211 We recorded the choice chamber in which each test bird first entered after the
212 opening. The use of first choice as a measure of the interest of birds to particular
213 chemical stimuli has been previously demonstrated (e.g. Bonadonna & Nevitt 2004;
214 Bonadonna et al. 2006). In order to minimize the duration of the trials and release the
215 birds as soon as possible, if after one minute the test bird had not left the central
216 chamber (20 of 39 birds), we then gently knocked on the middle of the entry door of the

217 central chamber to stimulate it to move to one of the choice chambers. Before knocking
218 the door, birds were previously orientated to, i.e. they were looking at, the choice
219 chamber they entered when we knocked the door. The knocking on the door did not
220 influence the preference of birds (see Results). The mean duration of the trials was 5
221 min 49 s.

222 Except for the first pair of birds each day, birds were first used as experimental
223 individuals and after that, they were used as scent donors. Each pair of donors were
224 used twice, one to test an experimental male and then to test an experimental female.
225 We balanced the side of the chamber where males and females were located. Birds were
226 released as soon as they were tested. The olfactometry device was carefully cleaned
227 with alcohol between trials.

228

229 CHEMICAL ANALYSIS

230

231 The entire available uropygial secretion from each bird was extracted with 200 μ l
232 dichloromethane and homogenised with a vortex mixer. The supernatant was transferred
233 to another glass chromatographic vial for chemical analysis.

234 A 450 GC (Varian) gas chromatograph was used, fitted with a CombiPal (CTC
235 Analytics) automatic injector and connected to a 240 MS (Varian) Ion Trap mass
236 spectrometer. A 1 μ l volume of the supernatant was injected splitless into a fused silica
237 FactorFour VF5ms capillary column (Varian) (30m, 0.25mm i.d., 0.25 μ m film
238 thickness). The injector, transfer line and ion source temperatures were 250, 280 and
239 240 °C, respectively. Helium was used as the carrier gas at a flow-rate of 1ml min⁻¹ and
240 oven temperature was programmed starting at 40 °C (1 min.), ramp at 7 °C min⁻¹ to 250
241 °C (5 min), ramp at 20 °C min⁻¹ to 300 °C where it was held for 5 min. A scan rate of

242 0.5 s/scan was employed, recording from 30 to 650 m/z in electron impact mode,
243 starting 3.5 min after injection.

244 Tentative identification of the compounds was first carried out by comparison
245 with those available in the NIST library. Then commercial standards, with purities \geq
246 90%, were used and positive identification of all the volatile compounds was confirmed
247 by coincidence of spectra and retention times. Quantitative analysis was carried out with
248 calibration curves prepared with the standards in dichloromethane.

249

250 DATA ANALYSIS

251 Behavioural study

252 To analyse whether birds could discriminate the scent of conspecifics by using chemical
253 cues alone, we performed a generalized linear mixed model with binomial errors and a
254 logit link function (GLMM). We modelled the probability that birds chose the scent of a
255 conspecific of the opposite sex from the scent of a conspecific of the same sex (as a
256 dichotomous variable: opposite sex (yes) versus same sex (not)) in relation to the sex of
257 the experimental bird, the side of the chamber where a particular sex was placed and
258 whether the experimental bird left the chamber when we opened the doors or after one
259 minute as fixed factors. We included the pair of donor birds in the model as a random
260 factor to control for the fact that pairs of donors were used twice.

261

262 Chemical analysis

263 As the volume of the uropygial gland secretion that we extracted differed among birds,
264 we calculated the proportion of each compound in the uropygial gland secretion. We
265 used the compositional analysis, consisting in logit-transforming the proportion data by
266 taking the natural logarithm of proportion/ (1 - proportion) to correct the problem of

267 non-independence of proportions (Aebischer, Robertson & Kenward 1993). Two
268 compounds (2-methyl decanone and decanol) appeared only in two individuals and
269 were excluded from the statistical analyses. We used PERMANOVA test to analyse
270 whether the composition of the uropygial secretion varied in relation to the sex and the
271 reproductive period (mating vs. breeding) in adult starlings. In a second PERMANOVA
272 test we analysed differences in the composition of the secretion of starlings in relation
273 to their age (nestlings vs. adults). When the PERMANOVA yielded a significant result,
274 we proceeded to univariate Mann-Whitney U Tests. We corrected for multiple testing
275 using the algorithm developed by Benjamini & Hochberg (1995) to control the false
276 discovery rate (FDR). This method is more suitable to ecological research than the less
277 powerful and very conservative Bonferroni procedures (e.g. Roback & Askins 2005). A
278 prerequisite in order to wisely apply FDR or other multiple testing procedures, is to
279 define appropriate groups, or families of hypotheses (Benjamini & Hochberg 1995;
280 Roback & Askins 2005). In our study, three families of hypotheses can be
281 conservatively distinguished in relation to the composition of the uropygial gland
282 secretion; those concerning the effect of a) sex ($N = 14$ tests, all P values ≥ 0.046 not
283 significant after FDR control); b) reproductive periods ($N = 14$ tests, all P values \geq
284 0.01785 not significant after FDR control); and c) age ($N = 14$ tests, all P values ≥ 0.021
285 not significant after FDR control) on gland composition.

286 In order to determine the set of chemical compounds of the uropygial gland
287 secretion that allows for the best discrimination between the sexes, we performed a
288 Discriminant Analysis. First we performed a Principal Component Analysis (PCA) with
289 the chemical compound proportions to obtain factors that summarized the variance of
290 the chemical compounds of the uropygial gland secretion of adult starlings. Later, we
291 used Discriminant Analysis to classify the PCA-factors in relation to the sex of adult

292 starlings in order to identify the combination of chemical compounds that contribute
293 most to the sexual differences in chemical composition of the secretion.

294 Finally, to assess differences in the size of the uropygial gland in relation to sex
295 and reproductive period we performed a two-way ANOVA. In this model we entered
296 the interaction sex*reproductive period to test whether changes in the uropygial gland
297 size across the breeding season varied between males and females. We used
298 STATISTICA 8.0 for statistical analyses except for GLMM and PERMANOVA tests
299 that were performed with the software package R 2.13.1.

300

301 **Results**

302

303 BEHAVIOURAL STUDY

304

305 When offered the scent of a conspecific of the opposite sex and a conspecific of the
306 same sex, the choice of birds was determined by their sex ($Z = 2.87$, $P = 0.004$), with
307 females preferentially choosing the scent of the opposite sex and males choosing the
308 scent of the same sex, i.e., most birds (27/39) chose the side of the chamber containing
309 the male scent (Fig. 2). Neither the side of the chamber where the male was located ($Z =$
310 $- 0.64$, $P = 0.52$) nor the fact that birds had chosen as soon as the doors were opened
311 versus after one minute ($Z = 1.03$, $P = 0.30$) influenced the choice of starlings.

312

313 CHEMICAL MEASUREMENTS

314

315 Uropygial secretions of starlings are composed by linear alcohols and methyl-ketones
316 (see Tables 1 and 2).

317

318 Sexual and seasonal variation

319 The composition of the uropygial gland secretion of adult starlings differed
320 significantly between sexes ($Pseudo-F = 244.73$, $DF = 1$, $P = 0.001$) and reproductive
321 periods ($Pseudo-F = 165.70$, $DF = 1$, $P = 0.001$). The interaction between sex and
322 reproductive period was not significant ($Pseudo-F = -63.05$, $DF = 1$, $P = 1.00$). The
323 uropygial gland secretion of males contained higher relative proportion of alcohols than
324 the secretion of females, but differences only reached significance levels in 2-
325 pentadecanone, that was lower in males than in females (Table 1). During the mating
326 period, adults exhibited a lower proportion of the most abundant compound,
327 hexadecanol (Table 1), and greater concentrations of the rest of alcohols, including
328 heptadecanol that did not appear in the secretions during the rearing of nestlings (Table
329 1). When adult birds were rearing nestlings, they also exhibited a lower proportion of 2-
330 tridecanone (Table 1).

331 The Principal Component Analysis of the chemical compounds of the uropygial
332 gland secretion of adult starlings provided 3 factors that accounted for 83 % of the
333 variance (see Table 3). The Discriminant Analysis of such factors in relation to the sex
334 of starlings showed significant differences only in the first factor (Wilks' Lambda =
335 0.94, $F_{1,28} = 4.48$, $P = 0.04$), that accounted for 52 % of the variance (Table 3). The
336 chemical composition of the uropygial gland secretion of males exhibited greater
337 proportion of 2-methyl tridecanone and most alcohols, except hexadecanol, than
338 females (see Table 3). On contrast, females had greater proportion of hexadecanol and
339 2-methyl pentadecanone than males.

340 Also, the size of the gland that secreted the compounds varied between
341 reproductive periods ($F_{1,95} = 71.16$, $P < 0.0001$), with adult birds exhibiting larger

342 glands during the rearing of nestlings than during mating (Fig. 3). There were not sexual
343 differences in the size of the gland ($F_{1,95} = 0.90$, $P = 0.34$) and the interaction between
344 sex and reproductive period was not significant ($F_{1,95} = 1.88$, $P = 0.17$) either.

345

346 Age variation

347 Composition of the uropygial gland secretion of adults and nestlings differed
348 significantly ($Pseudo-F = 8.80$, $DF = 1$, $P = 0.001$). Nestlings exhibited greater
349 proportions of methyl-ketones in their secretions than adults, except for 2-tridecanone,
350 that was only detected in the secretions of adult birds. Differences were statistically
351 significant in 2-pentadecanone, 2-hexadecanone and 2-heptadecanone (Table 2).
352 Alcohols that differed between ages were tridecanol, hexadecanol, heptadecanol and
353 octadecanol (Table 2). The most abundant alcohol in the secretion, hexadecanol,
354 together with other alcohols like heptadecanol and octadecanol, were present in lower
355 proportions in the secretions of nestlings than in those of adults. In contrast, the
356 proportion of a more volatile alcohol, tridecanol, was greater in nestlings than in adults'
357 secretions.

358

359 **Discussion**

360

361 Our results show for the first time that a passerine species can discriminate the sex of
362 conspecifics by relying on chemical cues. Furthermore, we have found patent sexual
363 differences in the composition of the uropygial gland secretion of starlings, which
364 suggests that this secretion may have the potential to reveal the sex to conspecifics in
365 spotless starlings. Females and males preferentially chose the male-scented side of the
366 chamber. The results found for female starlings are in accordance with our expectations

367 and results found by Zhang *et al.* (2010) who showed that female budgerigars preferred
368 the scent of a male. Contrary to our expectations, males oriented towards male scents.
369 On the other hand, male budgerigars did not exhibit any preference (Zhang 2011). In
370 our study starlings were captured at the beginning of reproduction, when males often
371 engage in aggressive intrasexual encounters to obtain a cavity for breeding. Therefore,
372 the preference of males for the scent of another male can be explained in terms of
373 intrasexual competition. Similar results were obtained by Jones and collaborators (2004)
374 in a study with crested auklets. They found that although both sexes approached scented
375 male models more closely than controls, males responded more to scented male models
376 than females did, which was explained by intrasexual aggression, as crested auklets
377 males are often involved in territorial disputes to maintain the nest site (Hagelin 2007a).
378 Male mice are also attracted to scent marks of other males because they provide useful
379 information about the social dominance of rival males (Arakawa *et al.* 2008). Further
380 experimental research is needed to establish whether preferences for the scent of males
381 change during the non-reproductive period for testing this hypothesis. Conversely,
382 Bonadonna *et al.* (2009) found that Antarctic prions cannot distinguish the sex of a
383 conspecific through its odour during the incubation period despite the fact that they are
384 able to recognize the scent of their partner (Bonadonna & Nevitt 2004). However, if
385 chemical cues in Procellariiform birds signal reproductive status, as it happens in
386 starlings (see below), the absence of sex-recognition based on odour towards the sex of
387 the incubating birds may be due to the fact that incubating birds were not considered as
388 potential partners.

389 The lack of sexual differences in the uropygial gland size suggests that birds are
390 producing similar amounts of secretion. Therefore, preferences for the scent of males
391 may be due to sexual differences in composition of the gland secretion, with males

392 producing higher proportions of alcohols, except hexadecanol, and lower proportions of
393 methyl-ketones, significantly the 2-methyl pentadecanone, than females (see table 3).
394 On contrast, females had a higher proportion of 2-methyl decanones, especially the 2-
395 methyl tridecanone, and lower proportion of alcohols. Our results agree with previous
396 studies that have found sexual differences in the composition of the uropygial gland
397 secretion in other avian taxa (e.g. Jacob, Balthazart & Schoffeniels 1979, Piersma,
398 Dekker & Sinninghe Damsté 1999, Whittaker *et al.* 2010, Zhang *et al.* 2010, Mardon *et*
399 *al.* 2010). Despite these compounds were directly collected from the uropygial gland,
400 and carefully protected during transport and storage, it cannot be discarded that some
401 chemical compounds may have undergone some degradation during sample collection
402 and processing (although see Hagelin 2008). Also, when birds spread the secretion into
403 the plumage, the composition may slightly change due to natural degradation in the
404 feathers (Mardon *et al.* 2010). Therefore, further experimental studies are needed to
405 disentangle which compounds, or combination of compounds, are involved in the
406 observed discrimination of sex in starlings.

407 The composition of the uropygial gland secretion did also vary in relation to the
408 reproductive status of starlings. In the course of the breeding period, adults showed an
409 increase in the proportion of hexadecanol, with a corresponding decrease in the rest of
410 alcohols. There was not only a modification in the composition of the secretions but
411 also in the amount secreted, as they exhibited larger uropygial glands during the rearing
412 of nestlings. An increase in gland size during the breeding period has also been reported
413 in house sparrows *Passer domesticus* (Pap *et al.* 2010) and European hoopoes *Upupa*
414 *epops* (Martín-Vivaldi *et al.* 2009). Changes in the composition of uropygial gland
415 secretions in relation to the reproductive period have been previously observed in other
416 species (e.g. Kolattukudy, Bohnet & Rogers 1987, Piersma, Dekker & Sinninghe

417 Damsté 1999, Haribal *et al.* 2005; Soini *et al.* 2007, Martín-Vivaldi *et al.* 2010). This
418 change in the composition suggests that birds may potentially signal their reproductive
419 status via chemical cues, as it has long been demonstrated in vertebrates and
420 invertebrates (Thomas 2011). However, the increased secretion activity, indicated by
421 the larger gland sizes, as well as the changes in the chemical composition of the gland
422 secretion, may have other non-exclusive functions than to serve in chemical
423 communication (Steiger, Schmitt & Schaefer 2011). Indeed, these functions may be
424 especially important during incubation and nest rearing due to their antibacterial
425 properties (e.g. Martín-Vivaldi *et al.* 2009, 2010). Also, secretion may help to maintain
426 feather conditions (e.g. Giraudeau *et al.* 2010), and/or to enhance their colour (López-
427 Rull, Pagán & Macías Garcia 2010). Finally, secretion may function as chemical
428 defence against parasites (Douglas 2008; Møller, Erritzøe & Rózsa 2010), or predators
429 (e.g. Burger *et al.* 2004; Reneerken, Piersma & Damsté 2005).

430 Our results also show differences in the chemical composition of secretions in
431 relation to the age of birds, with 12-14 day-old nestlings, that are almost fully-feathered,
432 exhibiting lower proportions of the main compound found in adult secretions
433 (hexadecanol) and greater proportions of methyl-ketones compared to adults. These
434 differences could be attributed to differences in the diet (e.g. Sandilands *et al.* 2004a;
435 Thomas *et al.* 2010) or differences in the allocation of resources. This may happen if
436 some compounds are more costly to produce than others, as trade-offs between
437 investment in growth and other requirements are expected in nestlings growing under
438 intense sibling competition levels such as spotless starlings (Gil *et al.* 2010).

439 Uropygial gland secretions in spotless starlings could potentially function as a
440 chemical signal used in reproductive behaviour, as they differ between the sexes,
441 reproductive status and ages. We have shown that chemicals emitted by birds are sex

442 specific and further research is required to establish whether birds can use these
443 chemical cues to ascertain the age and reproductive status of conspecifics. The chemical
444 profile of secretion also seems to differ from that reported in other species (e.g. Haribal
445 *et al.* 2005; Haribal, Dhondt & Rodríguez 2009). Several species appear to share similar
446 compounds in the uropygial gland secretion that have also been found in the secretions
447 of other taxa, from insects to mammals, that seem to play a role in intraspecific
448 communication. However, all the avian species in which the chemical cues have so far
449 been analysed exhibit a species- specific blend of compounds. These differences
450 between species may play a role in species recognition and, therefore, they may
451 constitute the first step in the use of uropygial gland secretions in mate recognition.

452 In conclusion, our experimental study demonstrates that starlings are able to
453 discriminate the sex of conspecifics by using chemical cues alone. Differences in the
454 composition of the uropygial gland secretion between species, sexes, ages and
455 reproductive status suggest that the uropygial gland secretion may potentially function
456 as a chemical signal used in reproductive behaviour as it conveys information about the
457 donor of the scent which allows the receiver to recognize mates. This is just a first step
458 in the investigation of the role of odours in sex recognition and social communication.
459 Further research is needed to examine whether these chemical cues may also provide
460 information allowing avian receivers to evaluate potential mates, as it has been largely
461 demonstrated for other animal taxa (see Johansson & Jones 2007 for a review) and for
462 visual and auditory cues in birds. Indeed, recent findings have demonstrated that
463 semiochemical profiles were correlated with heterozygosity both in male and female
464 black-legged kittiwakes *Rissa tridactyla* setting the scenario for the existence of odour-
465 based mate choice in birds (Leclaire *et al.* in press). The possible use of chemical
466 signals in birds challenges the traditional thought that birds only cue on visual and

467 auditory signals while assessing mates and/or rivals (Hagelin 2007b). On contrast to
468 most visual cues, such as plumage coloration, which are dead tissues produced during
469 moulting and thus revealing former condition-dependence (Hill 2007), chemical cues
470 are constantly produced, thereby potentially functioning as short term reliable signals of
471 physiological status in a context of sexual selection. Therefore, chemical cues may
472 provide an accurate assessment of the present quality of potential partners, and
473 consequently, they may play a role in sexual selection in birds that has been hitherto
474 ignored by behavioural and evolutionary biologists.

475

476

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478

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486

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- 681
- 682

683 **Table 1.** Mean \pm SE proportion of the different compounds of the uropygial gland
684 secretion of male and female starlings during mating and breeding. Also, univariate
685 Mann-Whitney U Test results for differences between sexes and reproductive periods
686 are shown. Significant results are shown in bold after correcting for multiple testing to
687 control the false discovery rate (FDR).
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Sex**Mann-Whitney****Reproductive period****Mann-Whitney**Males
(N=7)Females
(N=25)*Z**P*Mating
(N= 9)Breeding
(N= 23)*Z**P***Methyl-ketones:**

2-Decanone

n.d.

< 0.01 ± 0.01

0.01 ± 0.01

n.d.

2-Undecanone

0.05 ± 0.02

0.06 ± 0.01

-1.37

0.17

0.07 ± 0.02

0.06 ± 0.01

0.57

0.57

2-Dodecanone

0.03 ± 0.01

0.05 ± 0.01

-1.12

0.26

0.06 ± 0.02

0.04 ± 0.00

1.49

0.14

2-Tridecanone

0.06 ± 0.03

0.05 ± 0.02

0.55

0.59

0.17 ± 0.02

n.d.

5.47

<0.0001

2-Pentadecanone

0.67 ± 0.15

1.19 ± 0.10

-2.26

0.024

0.68 ± 0.15

1.23 ± 0.10

-2.37

0.02

2-Hexadecanone

0.23 ± 0.02

0.25 ± 0.02

-0.02

0.98

0.33 ± 0.05

0.21 ± 0.01

2.37

0.02

2-Heptadecanone

0.28 ± 0.03

0.29 ± 0.03

0.21

0.84

0.38 ± 0.05

0.26 ± 0.02

2.37

0.02

Alcohols:

Decanol

n.d.

0.01 ± 0.01

0.03 ± 0.03

n.d.

Undecanol

0.36 ± 0.08

0.20 ± 0.05

1.94

0.05

0.48 ± 0.09

0.14 ± 0.03

3.49

0.0005

Dodecanol

0.74 ± 0.16

0.47 ± 0.08

1.58

0.11

1.00 ± 0.12

0.35 ± 0.06

3.81

0.0001

Tridecanol

3.71 ± 0.71

2.64 ± 0.26

1.62

0.11

4.46 ± 0.38

2.26 ± 0.23

3.92

<0.0001

Tetradecanol

3.18 ± 0.59

2.39 ± 0.28

1.21

0.23

4.47 ± 0.36

1.81 ± 0.15

4.30

<0.0001

Pentadecanol

11.06 ± 0.90

9.83 ± 0.73

0.62

0.54

13.41 ± 0.58

8.81 ± 0.63

4.00

<0.0001

Hexadecanol

74.36 ± 3.56

79.64 ± 1.72

-1.34

0.18

65.42 ± 0.76

83.60 ± 0.75

-4.34

<0.0001

Heptadecanol

2.04 ± 0.96

1.13 ± 0.42

0.78

0.44

4.73 ± 0.28

n.d.

5.47

<0.0001

Octadecanol

3.24 ± 0.85

1.80 ± 0.35

1.53

0.12

4.32 ± 0.59

1.25 ± 0.23

3.48

0.0005

n.d. not detected

706 **Table 2.** Mean \pm SE proportion of the different compounds of the uropygial gland secretion of
 707 nestling and adult spotless starlings. Also, univariate Mann-Whitney U Test results for differences
 708 between ages are shown. Significant results are shown in bold after correcting for multiple testing to
 709 control the false discovery rate (FDR).

	Nestlings (<i>N</i> = 15)	Adults (<i>N</i> = 32)	Mann-Whitney	
			<i>Z</i>	<i>P</i>
Methyl-ketones:				
2-Decanone	n.d.	< 0.01 \pm 0.01		
2-Undecanone	0.05 \pm 0.02	0.06 \pm 0.01	1.23	0.22
2-Dodecanone	0.12 \pm 0.03	0.05 \pm 0.01	-1.91	0.06
2-Tridecanone	n.d.	0.05 \pm 0.01	2.24	0.02
2-Pentadecanone	10.88 \pm 4.79	1.08 \pm 0.09	-4.70	<0.0001
2-Hexadecanone	1.07 \pm 0.40	0.24 \pm 0.02	-2.78	0.005
2-Heptadecanone	6.54 \pm 4.15	0.29 \pm 0.02	-2.49	0.01
Alcohols:				
Decanol	n.d.	0.01 \pm 0.01		
Undecanol	0.24 \pm 0.22	0.23 \pm 0.04	2.96	0.003
Dodecanol	0.97 \pm 0.29	0.53 \pm 0.07	-1.05	0.30
Tridecanol	5.90 \pm 0.98	2.88 \pm 0.26	-3.10	0.002
Tetradecanol	4.87 \pm 1.77	2.56 \pm 0.26	-1.26	0.21
Pentadecanol	11.17 \pm 1.67	10.10 \pm 0.60	-1.57	0.12
Hexadecanol	57.97 \pm 6.81	78.49 \pm 1.58	3.42	0.0006
Heptadecanol	n.d.	1.33 \pm 0.39	2.24	0.02
Octadecanol	0.23 \pm 0.16	2.12 \pm 0.34	3.86	0.0001

725 n.d. not detected

726

727 **Table 3.** Factor Loadings of the Principal Component Analysis of chemical compounds of the
 728 uropygial gland secretion of adult starlings. Loadings greater than 0.65 are marked in bold. The
 729 Discriminant Analysis showed that Factor 1 significantly contributed to the sexual differences in the
 730 composition of the secretion.

731

	Factor 1	Factor 2	Factor 3
Methyl-ketones:			
2-Undecanone	0,01	-0,17	-0,84
2-Dodecanone	0,02	0,05	-0,94
2-Tridecanone	0,81	0,50	-0,10
2-Pentadecanone	-0,69	0,57	0,16
2-Hexadecanone	0,33	0,90	0,12
2-Heptadecanone	0,18	0,93	0,02
Alcohols:			
Undecanol	0,88	0,12	0,22
Dodecanol	0,92	0,09	0,09
Tridecanol	0,86	0,21	0,07
Tetradecanol	0,92	0,23	-0,08
Pentadecanol	0,43	0,56	-0,33
Hexadecanol	-0,79	-0,41	0,29
Heptadecanol	0,85	0,34	-0,20
Octadecanol	0,70	-0,19	-0,40
Proportion of explained variance	52 %	18 %	13 %

732

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734 **Fig. legend**

735 **Fig. 1.** Olfactometry chamber. The solid arrows indicate the direction of air flow within the
736 chamber, whereas the dashed lines indicate the direction of opening of the two doors connected
737 with the two plastic chambers. See methods for further details.

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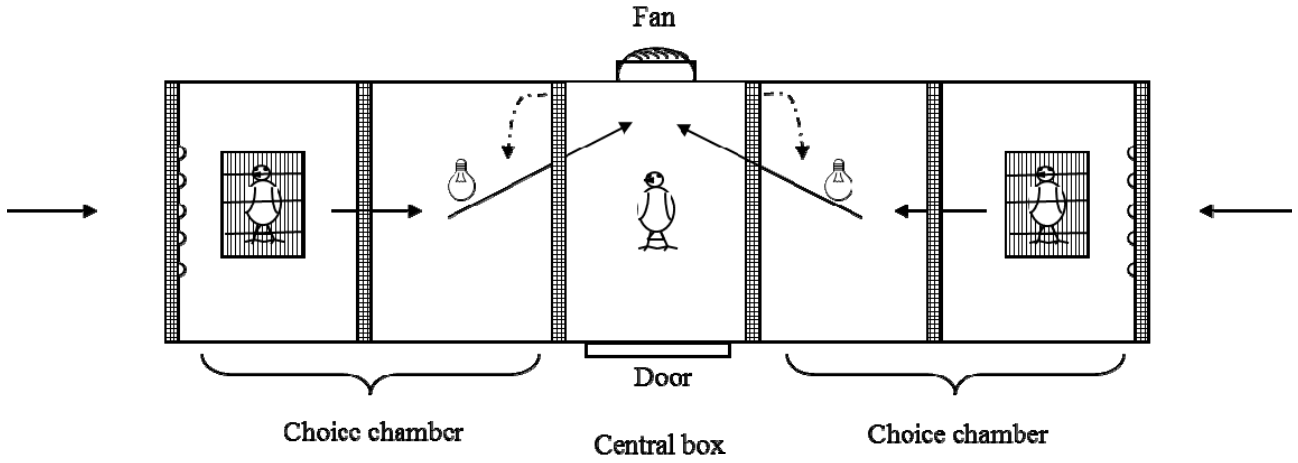
739 **Fig. 2.** Number of male (black) and female (white) adult spotless starlings that chose the side of the
740 chamber containing the scent of a male or a female starling. The horizontal line indicates the null
741 hypothesis (dashed for females and solid for males).

742

743 **Fig. 3.** Mean \pm SE uropygial gland size (mm^3) of adult spotless starlings during mating ($N = 10$)
744 and during the rearing of nestlings (breeding) ($N = 89$).

745

746 Fig. 1

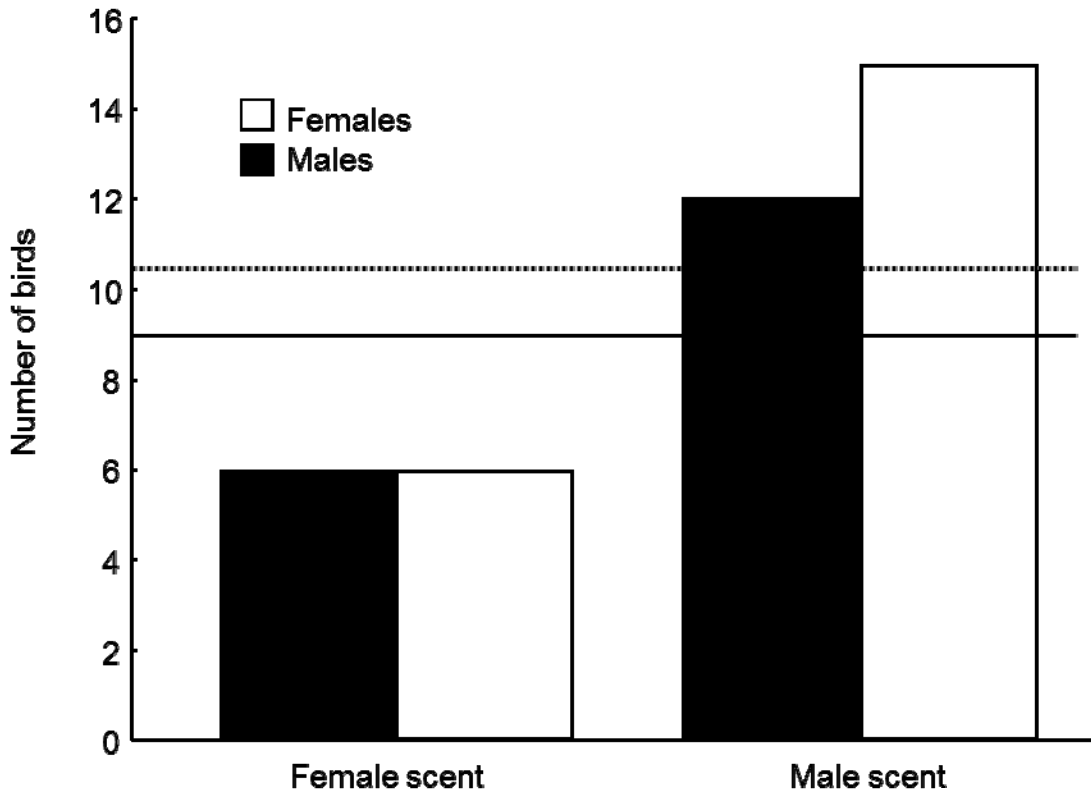


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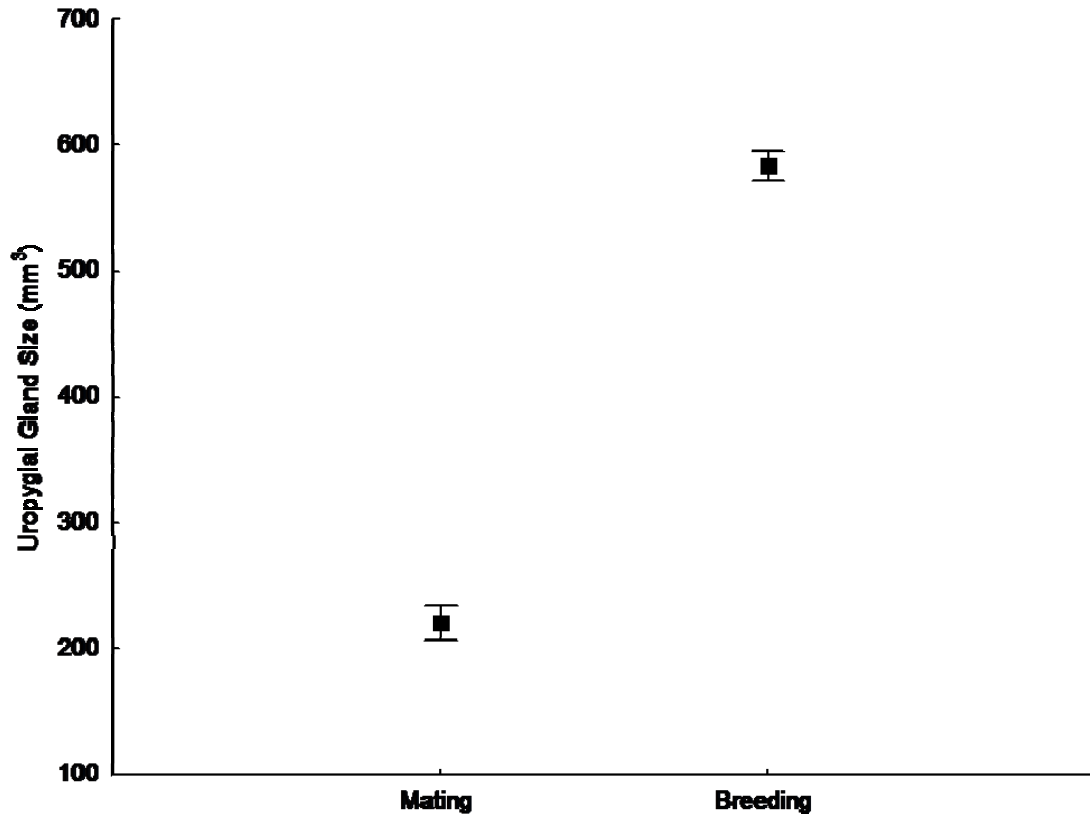
750 Fig. 2



751

752

753 Fig. 3



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