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

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Running headline: Odour-based sex recognition in a bird

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

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

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

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

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

Introduction

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

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

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

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

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

STUDY SPECIES

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

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

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

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

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

BEHAVIOURAL STUDY

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

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

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

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

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

CHEMICAL ANALYSIS

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

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

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

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

DATA ANALYSIS

Behavioural study

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

Chemical analysis

As the volume of the uropygial gland secretion that we extracted differed among birds, we calculated the proportion of each compound in the uropygial gland secretion. We used the compositional analysis, consisting in logit-transforming the proportion data by 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 $\geq$ 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 $\geq$ 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

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

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

Results

BEHAVIOURAL STUDY

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

CHEMICAL MEASUREMENTS

Uropygial secretions of starlings are composed by linear alcohols and methyl-ketones (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

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

Age variation

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

Discussion

Our results show for the first time that a passerine species can discriminate the sex of conspecifics by relying on chemical cues. Furthermore, we have found patent sexual differences in the composition of the uropygial gland secretion of starlings, which suggests that this secretion may have the potential to reveal the sex to conspecifics in spotless starlings. Females and males preferentially chose the male-scented side of the 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

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

The composition of the uropygial gland secretion did also vary in relation to the reproductive status of starlings. In the course of the breeding period, adults showed an increase in the proportion of hexadecanol, with a corresponding decrease in the rest of alcohols. There was not only a modification in the composition of the secretions but also in the amount secreted, as they exhibited larger uropygial glands during the rearing of nestlings. An increase in gland size during the breeding period has also been reported in house sparrows *Passer domesticus* (Pap *et al.* 2010) and European hoopoes *Upupa epops* (Martín-Vivaldi *et al.* 2009). Changes in the composition of uropygial gland secretions in relation to the reproductive period have been previously observed in other 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

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

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

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

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683 **Table 1.** Mean ± 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).
688

	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

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

	Nestlings (N= 15)	Adults (N = 32)	Mann-Whitney	
			Z	P
Methyl-ketones:				
2-Decanone	n.d.	< 0.01 ± 0.01		
2-Undecanone	0.05 ± 0.02	0.06± 0.01	1.23	0.22
2-Dodecanone	0.12 ± 0.03	0.05 ± 0.01	-1.91	0.06
2-Tridecanone	n.d.	0.05 ±0.01	2.24	0.02
2-Pentadecanone	10.88 ± 4.79	1.08 ± 0.09	-4.70	**<0.0001**
2-Hexadecanone	1.07 ± 0.40	0.24 ± 0.02	-2.78	**0.005**
2-Heptadecanone	6.54 ± 4.15	0.29 ± 0.02	-2.49	**0.01**
Alcohols:				
Decanol	n.d.	0.01 ± 001		
Undecanol	0.24 ± 0.22	0.23 ± 0.04	2.96	**0.003**
Dodecanol	0.97 ± 0.29	0.53 ± 0.07	-1.05	0.30
Tridecanol	5.90 ± 0.98	2.88 ± 0.26	-3.10	**0.002**
Tetradecanol	4.87 ± 1.77	2.56 ± 0.26	-1.26	0.21
Pentadecanol	11.17 ± 1.67	10.10 ± 0.60	-1.57	0.12
Hexadecanol	57.97 ± 6.81	78.49 ± 1.58	3.42	**0.0006**
Heptadecanol	n.d.	1.33 ± 0.39	2.24	**0.02**
Octadecanol	0.23 ± 0.16	2.12 ± 0.34	3.86	**0.0001**

n.d. not detected

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

733

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.

738

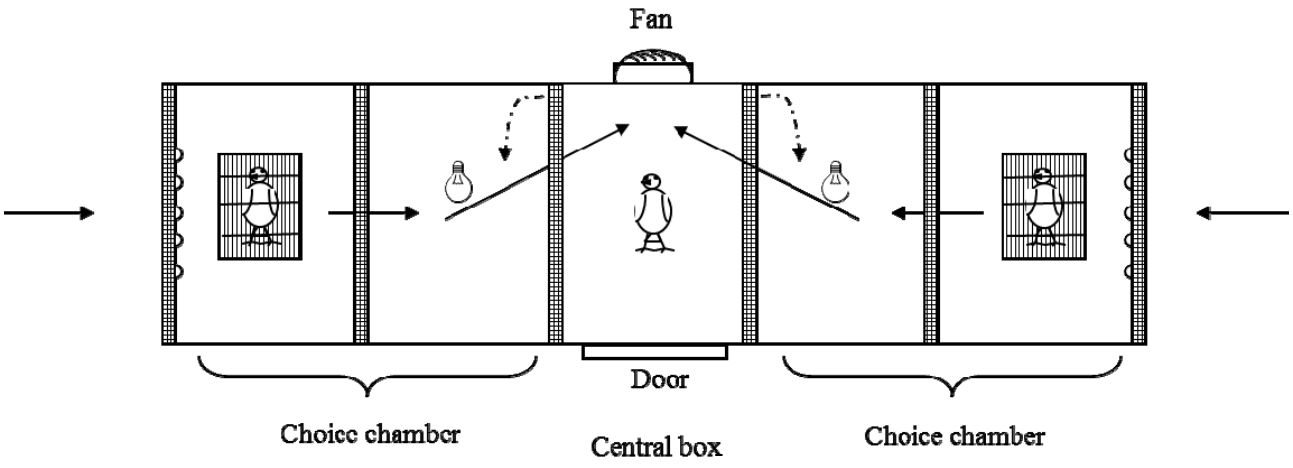
739 **Fig. 2.** Number of male (black) and female (white) adult spotless starlings that chose the side of the
740 chamber containing the scent of a male or a female starling. The horizontal line indicates the null
741 hypothesis (dashed for females and solid for males).

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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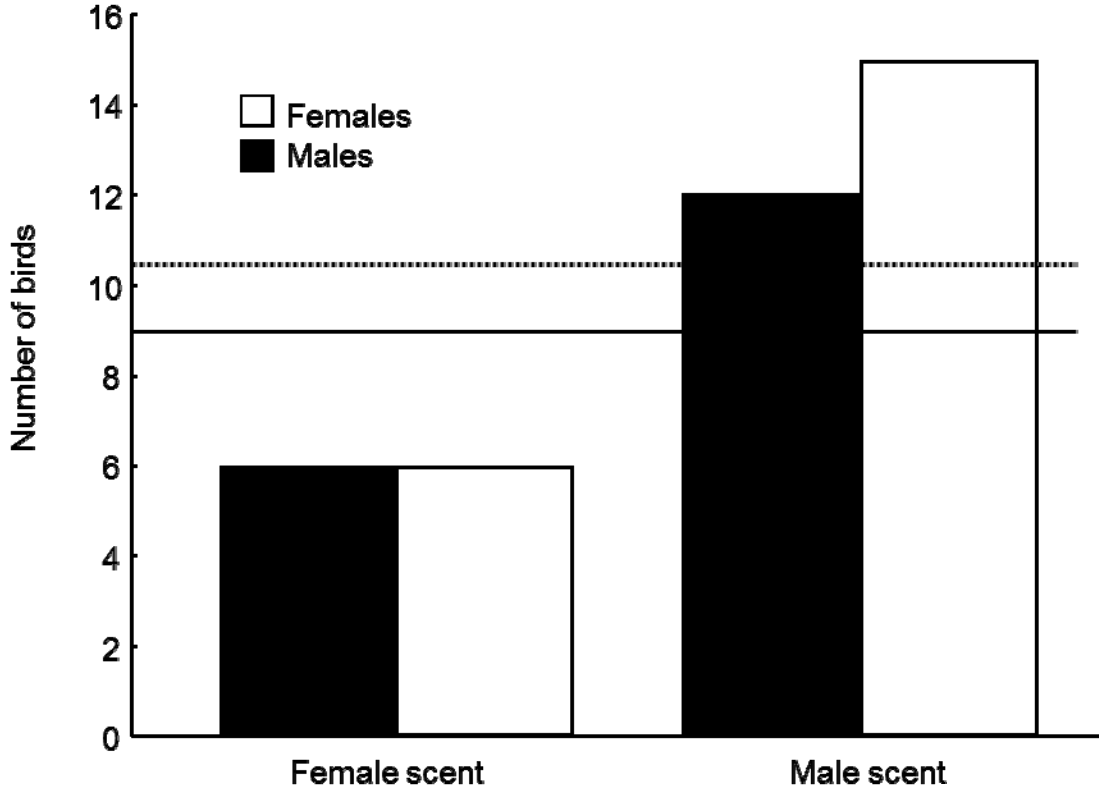
749

750 **Fig. 2**



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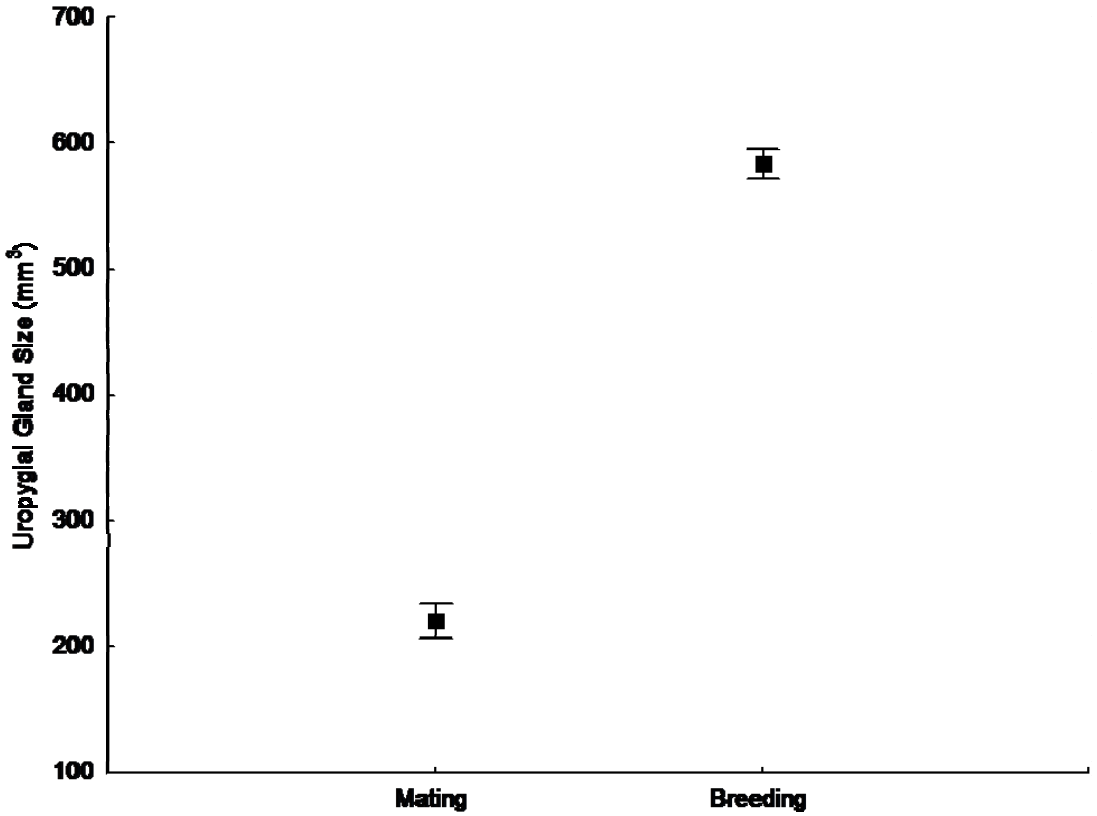
753 **Fig. 3**



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