No sex difference in preen oil chemical composition during incubation in Kentish plovers

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Preen oil – the secretion from the uropygial gland of birds – may have a specific function in incubation. Consistent with this, during incubation, the chemical composition of preen oil is more likely to differ between sexes in species where only one sex incubates than in species where both sexes incubate. In this study, we tested the generality of this apparent difference, by investigating sex differences in the preen oil composition of a shorebird species, the Kentish plover (*Anarhynchus*, formerly *Charadrius*, *alexandrinus*). As both sexes incubate in this species, we predicted the absence of sex differences in preen oil composition during incubation. In the field, we sampled preen oil from 9 females and 11 males during incubation, which we analysed with gas chromatography–mass spectrometry (GC–MS). Consistent with predictions, we found no sex difference in preen oil composition, neither in beta diversity (Bray-Curtis dissimilarities) nor in alpha diversity (Shannon index and number of substances). In Kentish plovers, the preen oil secreted during incubation may serve olfactory crypsis and protection against ectoparasites, as both functions received empirical evidence from studies on other shorebird species. Preen oil may also be involved in olfactory signalling (e.g. for mate choice, parent-offspring communication), but this possibility remains to be investigated.

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20   **Abstract**

21   Preen oil – the secretion from the uropygial gland of birds – may have a specific function in

22   incubation. Consistent with this, during incubation, the chemical composition of preen oil is

23   more likely to differ between sexes in species where only one sex incubates than in species

24   where both sexes incubate. In this study, we tested the generality of this apparent difference, by

25   investigating sex differences in the preen oil composition of a shorebird species, the Kentish

26   plover (*Anarhynchus*, formerly *Charadrius*, *alexandrinus*). As both sexes incubate in this

27   species, we predicted the absence of sex differences in preen oil composition during incubation.

28   In the field, we sampled preen oil from 9 females and 11 males during incubation, which we

29   analysed with gas chromatography–mass spectrometry (GC–MS). Consistent with predictions,

30   we found no sex difference in preen oil composition, neither in beta diversity (Bray-Curtis

31   dissimilarities) nor in alpha diversity (Shannon index and number of substances). In Kentish

32   plovers, the preen oil secreted during incubation may serve olfactory crypsis and protection

33   against ectoparasites, as both functions received empirical evidence from studies on other

34   shorebird species. Preen oil may also be involved in olfactory signalling (e.g. for mate choice,

35   parent-offspring communication), but this possibility remains to be investigated.

36

37   **Keywords**

38   Avian scent, bird odour, *Charadriiformes*, chemical profile, chemical camouflage, olfactory

39   communication, sex semiochemical, uropygial gland secretion, wader

40

41   **Introduction**

42   Most birds possess a sebaceous gland at the base of the tail – the uropygial gland (or preen

43   gland) – that produces an oily secretion (preen oil) (Jacob and Ziswiler 1982). Birds spread preen

44   oil over their plumage during preening (Moreno-Rueda 2017). The chemical composition of

45   preen oil typically consists of wax esters and other substances, such as alcohols, aldehydes,

46   alkanes, carboxylic acids and ketones (reviewed in Campagna *et al.* 2012, Alves Soares *et al.*

47   *accepted*). Preen oil is multifunctional, serving plumage maintenance, protection against

48   ectoparasites (e.g. feather degrading bacteria, eggshell bacteria, chewing lice) and waterproofing

49   (reviewed in Moreno-Rueda 2017). Preen oil is also an important source of body odour in birds

50   (Hagelin and Jones 2007) and may have odour-related functions, namely olfactory crypsis and

51   olfactory communication (reviewed in Grieves *et al.* 2022).

52   The first step to investigate the potential function(s) of preen oil is to describe the

53   variation in its chemical composition, notably seasonal changes and sex differences (Grieves et

54   al. 2022). In many species, preen oil composition changes during breeding, specifically at the

55   time of incubation and specifically in the incubating sex (Reneerkens et al. 2007), strongly

56   suggesting that preen oil has a function associated to incubation. First, a function of preen oil

57   during incubation could be protection against ectoparasites, in case incubating birds are exposed

58   to high parasitic loads in the nest or to limit pathogenic infection of the eggs. This was shown in

59   Eurasian hoopoes (*Upupa epops*) where only females (incubating sex) produce a dark preen oil

60   that contains antibacterial substances during incubation, and that is smeared on the eggs to

61   protect embryos from eggshell bacteria (Martín-Vivaldi et al. 2009, 2010). Second, a function of

62   preen oil during incubation could be olfactory crypsis, in case the incubating birds (and their

63   clutch or brood) are exposed to olfactorily searching nest predators (Reneerkens 2005). This is

64   the case in shorebirds (order Charadriiformes), where preen oil composition shifts from

65 monoesters (more volatile) to diesters (less volatile) during incubation (seasonal change in preen

66 oil; documented in 19 sandpiper, 6 plover and 1 oystercatcher species; Reneerkens *et al.* 2006),

67 solely in the incubating sex (sex-specific seasonal change in preen oil; documented in 7

68 sandpiper species; Reneerkens *et al.* 2007). The diester preen oil secreted during incubation is

69 less volatile than the monoester preen oil, which makes the incubating birds (or their clutch or

70 brood) less detectable to olfactorily searching nest predators (e.g. dog, Reneerkens 2005).

71 Finally, a function of preen oil during incubation could be olfactory intraspecific signalling (e.g.

72 for mate choice, "sex semiochemical hypothesis", Grieves *et al.* 2022). For example, in three

73 passerine species (order Passeriformes), preen oil composition differs between sexes during

74 breeding (Whittaker et al. 2010, Amo et al. 2012, Grieves et al. 2019a), which allows birds to

75 discriminate the sex of conspecifics by smell (Whittaker et al. 2011, Amo et al. 2012, Grieves et

76 al. 2019b).

77 Although several shorebird species (order Charadriiformes) have been studied with

78 regard to sex differences in preen oil (14 species), they were all studied using a fairly

79 straightforward analytical method (Reneerkens et al. 2002, 2007). This method consists in

80 describing preen oil composition using a single categorical variable (i.e. ester composition) with

81 three categories (i.e. monoesters only, mixture of monoesters and diesters, diesters only).

82 Reducing the complexity of preen oil composition (usually hundreds of substances) to a single

83 categorical variable is simple but effective. Indeed, this method revealed striking sex differences

84 in preen oil during incubation in uniparentally incubating species (diesters in the incubating sex,

85 monoesters in the non-incubating sex), but not in biparentally incubating species (diesters in both

86 sexes). However, subtle sex differences in biparentally incubating species may have been missed

87   using this categorisation, and may be uncovered using more advanced methods (e.g. multivariate

88   analyses).

89   In this study, we sampled preen oil from female and male Kentish plovers (*Anarhynchus*

90   *alexandrinus,* formerly *Charadrius alexandrinus*) during incubation, analysed their chemical

91   composition using GC–MS, and tested for sex differences in alpha and beta diversity using

92   multivariate statistical analyses. Given that both sexes incubate in this species (Kosztolányi and

93   Székely 2002), and assuming that this species undergoes the same sex-specific seasonal changes

94   in preen oil composition as the other shorebird species studied (Reneerkens et al. 2002, 2006,

95   2007), we predicted an absence of sex differences in preen oil composition during incubation.

96   Alternatively, sex differences in preen oil composition can be expected in case of sexual

97   selection or other sex-dependent reason. It should be emphasized that, since we sampled preen

98   oil only during the incubation period, our aim was not to investigate sex-specific seasonal

99   changes and replicate studies from Reneerkens *et al.* (2002, 2007). Rather, we used their findings

100   to make predictions on whether we should find sex differences during incubation in Kentish

101   plovers.

102

103   **Methods**

104   *Study site and species*

105   Fieldwork was conducted on breeding Kentish Plovers at the Samouco saltpans complex

106   (38°44′N, 8°59′W) on the south bank of the Tagus estuary, Portugal. In the study site, Kentish

107   Plovers breed on dykes of abandoned saltpans, nesting in the ground sparsely covered by

108   pebbles, wooden planks and salt marsh vegetation, isolated or in proximity to nests of black-

109   winged stilts *Himantopus himantopus* and little terns *Sternula albifrons* (Rocha et al. 2016). The

110   population (20-76 breeding pairs) is resident and presents an extended breeding season, from

111   early March, when males start to defend nesting sites, to the end of July. During the breeding

112   season, mates generally re-nest with a different mate (sequential polygamy), but monogamy is

113   also observed. Both parents incubate the eggs for a period of 25-26 days (Kosztolányi and

114   Székely 2002).

115   *Field methods*

116   As part of a colour-ringing marking program, from May to June 2019, Kentish Plovers were

117   caught on their nests using walk-in funnel traps (Székely et al. 2008). The birds were sexed using

118   plumage characteristics, measured and ringed (Székely et al. 2008). We collected preen oil from

119   20 birds (9 females and 11 males) by gently massaging the preen gland papilla with a 100 µl

120   microcapillary and snapping the end of the microcapillary (containing the extracted preen oil) in

121   a 2 ml glass vial with Teflon seal (Rotilabo ®) while wearing nitrile gloves. For some breeding

122   pairs, both partners of a breeding pair could be sampled (N = 8 samples from 4 pairs), but for

123   most pairs only a single bird was sampled (N = 12 samples). Samples were stored at –20 °C

124   during seven months, before being transferred at –80 °C for seven months until analysis. Bird

125   capture and sampling were carried out in accordance with the Portuguese Institute of Nature

126   Conservation and Forestry (ICNF) guidelines (license N°1/2019) and no additional institutional

127   animal care approval was required. To ensure the well-being of the birds, we took all necessary

128   measures to minimize any stress caused by capture and handling. After capture, birds were

129   placed inside a dark cotton bag before being ringed, measured and sampled. This procedure took

130   less than ten minutes per bird. Birds were released immediately after sampling, they showed no

131   sign of discomfort or stress (e.g. increased respiratory rate, open mouth breathing, or closed

132   eyes) and returned to incubate at their nest a few minutes after release.

133   *Chemical analysis*

134   All samples were first defrosted and then extracted by adding 500 µl of dichloromethane as a

135   solvent to the vials containing the microcapillary and the preen oil. After briefly vortexing each

136   sample, we transferred 100 µl of the solution (preen oil and dichloromethane) into a glass vial (2

137   ml, Rotilabo ®) containing a 100 µl glass inset, using a blunt point glass syringe (which was

138   washed with dichloromethane between each sample). For chemical analyses, we performed GC-

139   MS, using a gas-chromatograph (GC-2030, Shimadzu, Kyoto, Japan) equipped with a VF-5ms

140   capillary column (30 m x 0.25 mm ID, DF 0.25, 10 m guard column, Varian Inc., Lake Forest,

141   USA) and helium (at a 1 ml/min flow rate) as a carrier gas, coupled to a mass spectrometer

142   (GCMS-QP2020NX, Shimadzu) in split (1/10) mode. The settings for the gas chromatography

143   were as follows; injection temperature: 310 °C, starting temperature: 150 °C, followed by an

144   increase in temperature of 20 °C per min until reaching 280 °C, followed an increase of 5 °C per

145   minute until reaching the end temperature of 310 °C, which was kept for 20 min. For the mass

146   spectrometry, ion source temperature was set at 230 °C and interface temperature at 310 °C.

147   Seven GC blank samples (containing dichloromethane only) were analysed among the preen oil

148   samples.

149   *Chromatographic data processing*

150   GC-MS produces chromatograms, where each peak is a substance (defined by its retention time)

151   and the area of the peak represents the abundance of the substance (see Fig. 1 for a typical

152   chromatogram of Kentish Plover preen oil). We assumed that each peak represents a single

153 substance, but we acknowledge that a single peak can represent multiple substances that coelute

154 (i.e. with the exact same retention time). We extracted peak retention times and areas from

155 chromatograms using LabSolutions GCMS solution v4.52 (Shimadzu). Because the retention

156 time of a substance can vary subtly between samples, we aligned the chromatograms using the

157 *GCalignR* package (Ottensmann et al. 2018). We used the 20 preen oil samples and the seven

158 GC blank samples for the alignment. Substances detected in GC blank samples, as well as

159 substances detected in single samples, were removed to control for potential contamination. We

160 verified the quality of the alignment with a shadeplot (Fig. S1) in PRIMER v7.0.20 (Clarke and

161 Gorley 2015). Because the amount of preen oil collected was not standardized, we used relative

162 abundances (i.e. peak area divided by total chromatogram area) for the analysis. As we have no

163 prior knowledge about the substances potentially involved in sex differences, we log-transformed

164 (log(X+1)) the relative abundances, thereby increasing the weight of low-abundance substances

165 in the analysis (Clarke et al. 2014). We calculated the chemical diversity (Shannon index) and

166 richness (number of substances) of each sample, as measures of alpha diversity, using the *vegan*

167 package (Oksanen et al. 2019). If the detectability of substances was positively correlated to their

168 retention time, there could be a methodological issue (e.g. more volatile substances evaporating

169 or breaking more than less volatile substances). We verified that this was not the case, as the

170 effect of the retention time on the detectability of substances was not positive linear (polynomial

171 beta regression: $\beta = 0.54$, $P = 0.48$), but negative quadratic (polynomial beta regression: $\beta = -$

172 5.57, $P < 0.001$). This shows that both more volatile and less volatile substances were less

173 detected than substances with intermediate retention time (Fig. S2). All the chromatographic data

174 processing was conducted in R v4.2.2 (R Core Team 2022) and is detailed in an R Markdown

175 document (Baumer and Udwin 2015) in the Supplemental Information.

176   An accurate identification of the substances would have required sophisticated analytical

177   methods, including calculating retention indices, comparing substances with commercially

178   available standards and using two columns of different polarity (e.g. Alves Soares *et al.* 2024).

179   For structural identification of esters, other methods could be conducted, such as combined GC

180   and GC-MS using synthesized standards (Sinninghe Damsté et al. 2000, Rijpstra et al. 2007). As

181   we were interested in quantitative, rather than qualitative, chemical differences, we did not need

182   to identify the substances and used retention times instead. We putatively identified the chemical

183   substances by comparing their mass spectrometry (MS) to that of the NIST library

184   (NIST/EPA/NIH Mass Spectral Library 2017) and recording the substance name with the highest

185   match, but this method is not accurate enough to identify substances with certainty. For this

186   reason, we do not provide the list of putative (and likely erroneous) substance names. However,

187   we recorded the class of the substances, in case the class of the putatively identified substances

188   was the same across all samples (see Table S1 for the list of substances, including retention

189   times, mean relative abundances and classes). The raw chromatographic data are available at the

190   repository PUB – Publications at Bielefeld University (link and DOI to be added upon

191   acceptance).

192   *Statistical analysis*

193   We tested for sex differences in preen oil composition using 20 samples (9 females, 11 males).

194   First, to test for sex differences in the overall chemical composition (i.e. beta diversity), we

195   performed a permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis

196   dissimilarities using the *adonis2* function from the *vegan* package (Oksanen et al. 2019). Bray-

197   Curtis dissimilarity is pertinent for the analysis of abundance data, notably because it ignores

198   joint absences (Clarke et al. 2014). PERMANOVA was run with 9,999 permutations and

199 sequential effects (type I sums of squares). As fixed effects, we included *sex*, but also *number of*

200 *days after laying* to test for a potential seasonal effect as preen oil composition can change over

201 short periods (less than a week, Grieves *et al.* 2022), and the interaction between *sex* and *number*

202 *of days after laying*, as seasonal changes may differ between sexes (Grieves et al. 2022). Some of

203 our samples were collected from breeding partners (N = 8 "paired" samples from 4 breeding

204 pairs), and preen oil can be more similar within than between breeding pairs (Gilles et al. 2024).

205 However, using blocking permutations within breeding pairs is not appropriate to deal with the

206 possible pseudoreplication in this dataset, because in 12 cases there is only one data point from a

207 pair (i.e. only one possible choice within a pair); therefore we applied an alternative approach.

208 First, we randomly excluded four of the "paired" samples so that we included only independent

209 samples (N = 16 samples, only one sample from a breeding pair). The four excluded samples

210 always included two females and two males so that the ratio between the sexes was not distorted

211 (seven females and nine males). Second, we ran iterated PERMANOVAs (1,000 iterations) on

212 the 16 samples randomly selected before each run. We report the median (and interquartile

213 range) of the SS, R^2 and F values from the iterated PERMANOVA runs. P values were

214 calculated as $P = \dfrac{\sum_{i=1}^{N_{iterations}}(1 + \sum_{j=1}^{N_{perm}}\mathrm{I}(F_j \ge F_{obs_i}))}{N_{iterations} \times (1 + N_{perm})}$ where $N_{iterations}$ is the number of iterations of

215 PERMANOVAs, N_{perm} is the number of permutations per PERMANOVA, F_j is the F-statistic

216 for the jth permutation, F_{obs_i} is the observed F-statistic in the ith iteration, and I(condition) is an

217 indicator function that equals 1 if the condition is true and 0 otherwise. We also tested for a sex

218 difference in dispersion (or variance) using the *betadisper* function from the *vegan* package

219 (Oksanen et al. 2019). We used non-metric multidimensional scaling (NMDS) plots for

220 visualization of differences in Bray-Curtis dissimilarity (beta diversity).

221   Second, to test for sex differences in chemical diversity (Shannon index) and richness

222   (number of substances) (i.e. alpha diversity), we performed linear models (LMM) using the *lmer*

223   function in the *lme4* package (Bates 2010). For both models, *sex* and *number of days after laying*

224   were included as fixed effects, and *pair ID* as random effect. Using *pair ID* as a random effect,

225   we controlled for the potential increased similarity within breeding pairs, and thus we could

226   include all 20 samples (nine females and eleven males). We assessed the significance of fixed

227   effects ($\alpha = 0.05$) by checking whether their 95% confidence interval (95% CI) contained zero,

228   using the *broom.mixed* package (Bolker et al. 2022). Assumptions of normality and

229   homoscedasticity of the residuals were verified using the *performance* package (Lüdecke et al.

230   2021). All plots were created with *ggplot2* (Wickham 2016), all analyses were performed in R

231   v4.2.2 (R Core Team 2022). Data and code are available in the Supplemental Information and at

232   the repository PUB – Publications at Bielefeld University (link and DOI to be added upon

233   acceptance).

234

235   **Results**

236   We detected a total of 95 chemical substances in the preen oil of Kentish plovers, with on

237   average 63 substances (SD = 9) per sample (on average 62 substances in females and 63

238   substances in males). These numbers should be treated as minima, as they are based on the

239   assumption that one peak represents one substance, but it is possible that one peak represents

240   multiple substances (in case of coeluting substances). Most putative substances appeared to be

241   monoesters, while no diester was detected (Table S1). About one third of the substances (32%, *N*

242   = 35 substances) were detected in all 20 samples, and no substance was sex-specific (i.e. detected

243   in females only or males only).

244   We found no sex difference in preen oil composition (beta diversity) based on Bray-

245   Curtis dissimilarities (PERMANOVA: $P = 0.35$, $R^2 = 0.11$). The absence of a sex difference can

246   be seen on the NMDS plot (Fig. 2a) where the 95% confidence intervals for each sex overlap

247   entirely. The preen oil composition of females and males also did not differ in dispersion ($P =$

248   0.39). In addition, no sex difference was detected in alpha diversity, neither in chemical diversity

249   (LMM: β [95% CI] = 0.09 [–0.15; 0.36], Fig. 2b) nor richness (LMM: β [95% CI] = 5.88 [–12.7;

250   26.4], Fig. 2c). Preen oil composition did not change over the course of incubation (from 1 day

251   until 33 days after laying), neither in Bray-Curtis dissimilarities (PERMANOVA: $P = 0.48$, $R^2 =$

252   0.11), diversity (LMM: β [95% CI] = 0.00 [–0.01; 0.01]), nor richness (LMM: β [95% CI] = 0.02

253   [–0.81; 0.86]). Finally, we detected no effect of the interaction between sex and the number of

254   days after laying, neither in Bray-Curtis dissimilarities (PERMANOVA: $P = 0.34$, $R^2 = 0.06$),

255   diversity (LMM: β [95% CI] = 0.00 [–0.02; 0.01]), nor richness (LMM: β [95% CI] = –0.32 [–

256   1.73; 0.93]). Detailed results are available in the supplemental information (Tables S2–3).

257

258   **Discussion**

259   As predicted, we found no sex difference in the preen oil of Kentish plovers during incubation,

260   neither in beta diversity nor in alpha diversity. This is consistent with previous findings that, in

261   shorebirds with biparental incubation, both sexes secrete a similar preen oil during incubation

262   (Reneerkens et al. 2007, Grieves et al. 2022). Using more advanced statistics than the classical

263   studies on the chemistry of the preen oil of shorebirds (Reneerkens et al. 2002, 2006, 2007), we

264   did not uncover subtle sex differences.

265   Our finding that both sexes secrete a similar preen oil during incubation may indicate a

266   specific function of preen oil in incubation, but only if preen oil composition changes

267   specifically during this period, as in other shorebird species (Reneerkens et al. 2002, 2006,

268   2007). Because we sampled preen oil only during the incubation period, we could not test for

269   such seasonal changes. We assumed that Kentish plover preen oil would follow the general

270   pattern identified by Reneerkens *et al.* (2002) in other shorebirds, that is a switch from

271   monoesters to diesters at the onset of incubation followed by a switch back to monoesters after

272   incubation. It seems however that Kentish plovers do not follow this general pattern. Indeed, our

273   putative identification of the class of substances revealed that the preen oil of incubating Kentish

274   plovers contained predominantly monoesters, and no diesters (Table S1). Although surprising,

275   this finding is consistent with a preliminary study (Reneerkens 2007), which found only

276   monoesters in the preen oil of incubating Kentish plovers (as well as in incubating Northern

277   Lapwings *Vanellus vanellus* and Eurasian dotterels *Anarhynchus morinellus*). Together, these

278   results suggest that, in some shorebird species including Kentish plover, preen oil composition

279   may not switch to a diester mixture during incubation, and challenge the idea that preen oil has a

280   role in incubation in these species. However, even if the preen oil of Kentish plovers does not

281   contain any diester during incubation, it may still undergo seasonal changes, although not as

282   dramatic as a complete shift to diesters. For example, preen oil may consist of a mixture of

283   monoesters year-round, but the monoesters produced during incubation may be less volatile than

284   those secreted the rest of the year. In any case, we call for caution with these preliminary results,

285   because the analytical methods used by Reneerkens (2007) (i.e. judging peak patterns from

286   chromatograms) and our study (i.e. comparing mass spectrometry with the NIST library) are

287   simplistic and prone to inaccuracies. This warrants a more accurate identification of the

288   substances in the preen oil of Kentish plovers (e.g. Rijpstra *et al.* 2007, Alves Soares *et al.* 2024),

289   using samples collected throughout the year.

290   From our descriptive results, we can only speculate on the function of preen oil during

291   incubation in Kentish plovers. Preen oil may have a role in olfactory crypsis at the nest, as

292   proposed for the 26 other shorebird species studied (Reneerkens et al. 2006). Kentish plovers

293   nest on the ground and are vulnerable to olfactorily searching nest predators, such as dogs, foxes,

294   snakes and lizards (Fraga and Amat 1996, Kosztolányi et al. 2009). When producing a low-

295   volatility preen oil, incubating birds (and/or their clutch or brood) may be less detectable to

296   olfactorily searching nest predators, thereby increasing nest survival (Reneerkens 2005, Grieves

297   et al. 2022). To further investigate this possibility, we should sample preen oil from Kentish

298   plovers across several breeding stages (not only during incubation) and measure its volatility. We

299   hypothesize that the preen oil from Kentish plovers undergoes similar shifts, from more volatile

300   substances to less volatile volatile substances at the onset of breeding and back to more volatile

301   substances at the end of breeding, like in the other shorebird species studied (Reneerkens et al.

302   2006).

303   In addition to olfactory crypsis, preen oil may have other roles during incubation. Preen

304   oil may protect incubating birds from feather degrading bacteria (e.g. red knots *Calidris canutus*,

305   Reneerkens *et al.* 2008; Eurasian hoopoes, Ruiz-Rodríguez *et al.* 2009) and their clutch from

306   eggshell bacteria (e.g. Eurasian hoopoes, Martín-Vivaldi *et al.* 2010). To test this, we should

307   assess the antimicrobial properties of the preen oil of Kentish plovers (e.g. Shawkey *et al.* 2003).

308   Preen oil may also have a role in chemical signalling for mate choice. Sex recognition based on

309   preen oil odours, like in dark-eyed juncos (*Junco hyemalis,* Whittaker *et al.* 2010, 2011) and

310   song sparrows (*Melospiza melodia*, Grieves *et al.* 2019a, b), is not likely in Kentish plovers

311 because of the absence of sex differences during incubation. To confirm this, we should also test

312 for sex differences in preen oil before incubation, when mate choice actually occurs. Also, preen

313 oil may have signalling roles other than sex recognition. Birds may display their genetic

314 compatibility (e.g. major histocompatibility complex) in their preen oil odours, like in black-

315 legged kittiwakes (*Rissa tridactyla*, Leclaire *et al.* 2014) and song sparrows (Grieves et al.

316 2019c), and they may use preen oil to assess relatedness of a potential mate (Krause et al. 2012,

317 Caspers et al. 2015a). It should be emphasized that preen oil could have a function for chemical

318 protection and chemical signalling at the same time. Indeed, preen oil odours could signal greater

319 protection of the offspring (e.g. against predators via olfactory crypsis, or against pathogens via

320 antimicrobial activity) and thereby be sexually selected signals.

321 We acknowledge that our negative results (absence of sex differences) may be due to the

322 limited sample size and thus limited statistical power (i.e. false negative, or type II error). To

323 evaluate whether our negative results are more likely false negatives or true negatives, we can

324 compare the effect sizes of positive results from other studies with the confidence intervals from

325 our study (Nakagawa and Cuthill 2007). A study on the preen oil composition of blue tits found a

326 significant sex difference in chemical richness, with females producing on average 38 substances

327 more than males (Caspers et al. 2022). This effect size (38 substances) falls well outside our

328 confidence interval ([−12.7; 26.4]), indicating that our study would have had the power to detect

329 such an effect. Although this does not prove that our results are true negatives, it gives us

330 confidence that they likely are. We note that we did not focus on the volatile fraction of preen

331 oil, which would be the most relevant to study for its putative odour-related roles, like olfactory

332 crypsis or chemical signalling. Instead, we analysed the whole preen oil composition, which

333 includes both volatile and nonvolatile compounds. We did so because nonvolatile compounds

334   may be precursors of volatile compounds involved in crypsis or signalling, and are thus also

335   relevant for such studies (Mardon et al. 2011). For example, the monoesters and diesters in the

336   preen oil of red knots *Calidris canutus* are nonvolatile but still seem to have different odours or

337   odour levels (different detection success of monoester and diester preen oil by a dog, Reneerkens

338   2005). Another example is the preen oil of song sparrows, where the sex differences in

339   nonvolatile esters seem to translate into sex differences in body odour, allowing the birds to

340   discriminate sex by smell (Grieves et al. 2019b).

341   Our results are based on a single species and a single period, and thus cannot elucidate

342   whether preen oil has a role (and which role) in incubation. However, our study provides

343   valuable data on sex differences in preen oil. To investigate the function of preen oil in Kentish

344   plovers, future studies should sample preen oil at different breeding stages (notably during mate

345   choice and non-breeding) and measure its volatility. Importantly, future studies should conduct

346   experiments, such as antimicrobial assays to test for antiparasitic protection (e.g. Shawkey *et al.*

347   2003, Martín-Vivaldi *et al.* 2010), detectability trials or field experiments to test for olfactory

348   crypsis (e.g. Reneerkens 2005, Selonen *et al.* 2022), and behavioural trials to test for olfactory

349   communication (e.g. Caspers *et al.* 2015b, Grieves *et al.* 2019b).

350

351   **Conclusion**

352   Sex differences in preen oil composition during incubation in Kentish plovers could not be

353   detected during incubation in Kentish plovers, a shorebird species in which both sexes incubate.

354   This result is consistent with previous studies, where sex differences in preen oil occurred during

355   incubation in uniparentally incubating species more than in biparentally incubating species. The

356   similar preen oil secreted by the female and the male during incubation may have a function for

357   olfactory crypsis, as proposed for other shorebird species, but also for protection against

358   ectoparasites and/or olfactory communication. Importantly, to elucidate whether preen oil has a

359   function, future studies should first test whether preen oil composition changes seasonally,

360   specifically at the time of incubation.

361

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367

368   **Data Availability**

369   Data and code used for the analyses are available in the Supplemental Information and at the

370   repository PUB – Publications at Bielefeld University (link and DOI to be added upon

371   acceptance).

372

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510

Figure 1

Representative chromatogram of the preen oil of Kentish Plovers.

The illustration depicts a female Kentish Plover with a zoom on its uropygial gland secreting preen oil.
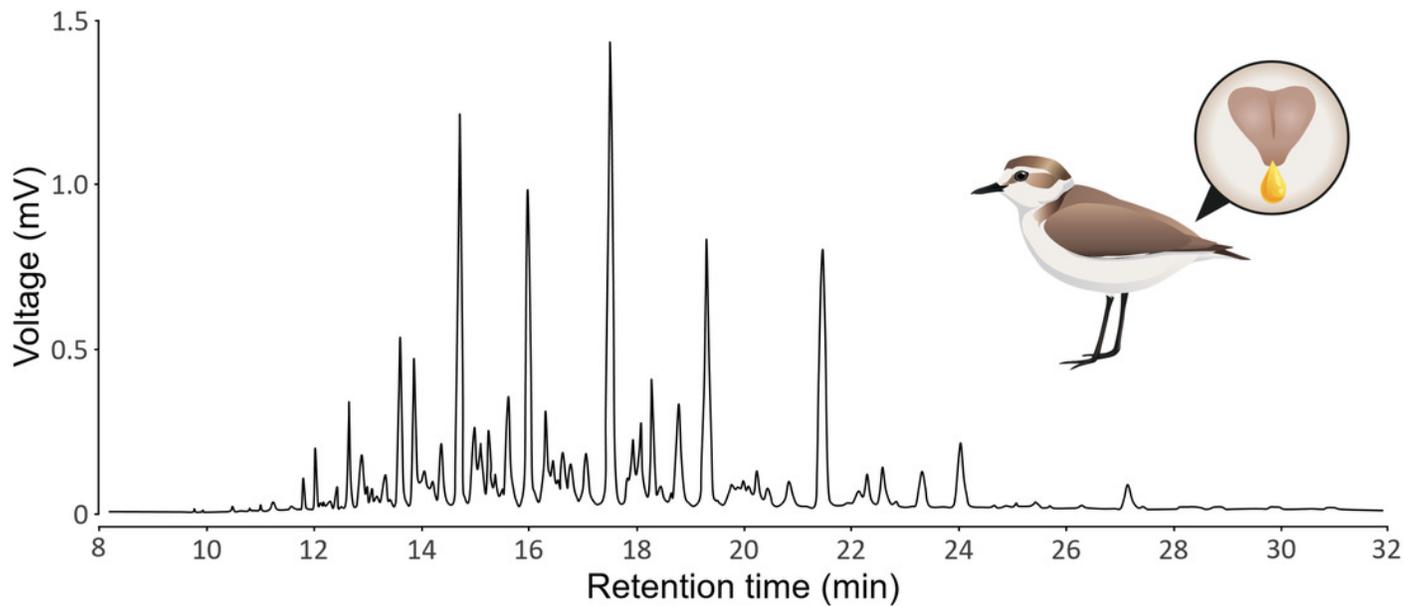
Figure 2

No sex difference in the preen oil composition of Kentish Plovers.

(a) Non-metric multidimensional scaling (NMDS) plot representing Bray-Curtis dissimilarity in chemical composition. 2D Stress measures the "goodness of fit" of the NMDS ordination, with a value < 0.1 indicating a good fit. The ellipses for each sex (95% confidence intervals assuming a multivariate t-distribution) overlap entirely, highlighting the absence of a sex difference in beta diversity. Besides, no sex difference was detected in alpha diversity, namely **(b)** chemical diversity (Shannon index) and **(c)** chemical richness (number of substances) of preen oil.