Diversity of compounds in femoral secretions of Galápagos iguanas (genera: *Amblyrhynchus* and *Conolophus*), and their potential role in sexual communication in lek-mating marine iguanas (*Amblyrhynchus cristatus*)

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Background. Chemical signals are widely used in the animal kingdom, enabling communication in various social contexts, including mate selection and the establishment of dominance. Femoral glands, which produce and release waxy secretions into the environment, are organs of central importance in lizard chemical communication. Galápagos marine iguanas (*Amblyrhynchus cristatus*) is a squamate reptile with lek-mating system. Although the lekking behavior of marine iguanas has been well-studied, their potential for sexual communication via chemical cues has not yet been investigated. Here we describe the diversity of the lipophilic fraction of males' femoral gland secretions among 11 island populations of marine iguanas, and compare it with the composition of its sister species, the Galápagos land iguana (*Conolophus subcristatus*). We also conducted behavioural observations in marine iguana territorial males in order to explore the possible function of these substances in the context of male dominance in leks. **Methods.** Femoral secretions were analyzed by Gas Chromatography coupled to Mass Spectrometry (GC-MS), and Gas chromatographwitha flame ionizationdetector (GC-FID) in order to characterize the lipophilic composition. To understand the potential role of femoral secretions in marine iguana intraspecific communication, territorial males were sampled for their femoral glands and monitored to record their head bob rate – a territorial display behaviour in males – as well as the number of females present in their leks. **Results.** We found that the gland secretions were composed of ten saturated and unsaturated carboxylic acids ranging in chain lengths between C_{16} and C_{24}, as well as three sterols. Cholesterol was the main compound found. Intriguingly, land iguanas have a higher diversity of lipophilic compounds, with structural group of lipids (i.e. aldehydes) entirely absent in marine

iguanas; overall the chemical signals of both species were strongly differentiated. Lipid profiles also differed among populations of marine iguanas from different islands, with some islands demonstrating a high diversity of lipophilic compounds (i.e. full spectra of compounds), and others lacking one or more compounds. Among the compounds most frequently found missing were 11- and 13- eicosenoic acids. Gland secretions of males with a better body condition and with a higher dominance status (i.e. those accompanied by females and with higher head bob display) were proportionately richer in C_{20}-unsaturated fatty acids (11-eicosenoid acid). **Discussion.** Land and marine iguanas strongly diverged in their chemical composition of the femoral glands likely due to ecological differences between both species. Despite that marine iguana populations varied in their femoral gland composition that was not related to their genetic structure. Our results indicated that 11-eicosenoic acid may play an important role in intraspecific chemical communication in marine iguanas.

1 **Diversity of compounds in femoral secretions of Galápagos iguanas (genera:**

2 ***Amblyrhynchus* and *Conolophus*), and their potential role in sexual communication in lek-**

3 **mating marine iguanas (*Amblyrhynchus cristatus*)**

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INTRODUCTION

Intraspecific chemical communication is a widespread phenomenon among vertebrates (Wyatt 2003). Although reptiles rely heavily on visual signals to communicate with conspecifics, they also use chemical signals in sexual interactions, as well as in a broader social context (Mason & Parker 2010). Squamate reptiles (lizards and snakes) are very suitable models for researchers aiming to study chemical communication. This is mainly due to highly-developed chemosensory capacity, which is used for intraspecific communication. For instance, substrate-deposited scents are used by snakes to locate partners and/or avoid rivals (LeMaster & Mason 2003; Shine et al. 2005). Lizards have the ability to produce scent marks that are recognized by conspecifics (e.g. Carazo et al. 2008; Khannoon et al. 2011; Martins et al. 2006). The popularity of lizards as models to study chemical communication has been increasing steadily. However, the functions of the specific compounds that make up chemical signals remain poorly known.

One of the most important organs involved in chemical communication are femoral glands, which are epidermal skin glands situated ventrally on the hind limbs (Mayerl et al. 2015). Reptiles release waxy secretions from their femoral glands that are composed of a mixture of proteins and lipids (Weldon et al. 2008). For example, desert iguanas (*Dipsosaurus dorsalis*) have heavy femoral secretions, composed of a 20% lipid fraction and 80% of protein material (Alberts 1990). Proteins absorb ultraviolet light that could be used by desert iguanas to locate pheromones of low volatility (Alberts 1989). Inter-species variability in proteins from femoral glands suggests that proteins could be used for species discrimination in sympatrically occurring lizard species (Alberts 1991). A recent study on Turks and Caicos iguanas (*Cyclura carinata*) showed that genetic differentiation among lineages from different islands is reflected by the pattern of proteins in femoral secretions (Welch et al. 2017). In addition, the lipophilic compounds in the femoral secretions play an important role in many types of sexually-mediated behaviour. For example, femoral glands in males of some lizard species emit chemicals that could communicate specific information about the traits of the signaller, and may therefore guide female mate choices (López & Martín 2005; Martín & López 2006; Martín & López 2011; Olsson et al. 2003). A link between chemical signals and dominance has also been suggested. For instance, femoral gland secretions of male Ibero*lacerta monticola* convey information about the individual's dominance status (Moreira et al. 2006). This situation is hardly surprising, given

90   that the function and activity of femoral glands are regulated by androgens (Fergusson et al.
91   1985). In green iguanas, *Iguana iguana*, the level of testosterone is positively correlated with the
92   amount of lipids in the femoral secretions of dominant males (Alberts et al. 1992). In this
93   framework, several studies have described the lipid components of the femoral glands in various
94   species of lizards (reviewed in (Martín & López 2014; Weldon et al. 2008)). Although most
95   research on the specific chemicals that compose femoral secretions has focused on lacertids
96   (Heathcote et al. 2014; Martín et al. 2015; García-Roa et al. 2017), a few older studies about
97   iguanids are also available (Alberts 1990; Alberts et al. 1992; Weldon et al. 1990). The femoral
98   secretions of the green iguana *I. iguana* are known to contain a high diversity of volatile lipids
99   that appear to be received by chemoreception (Alberts 1993; Alberts et al. 1992). These lipids
100   include several saturated and unsaturated fatty acids of different chain lengths (C_{14}-C_{26}), as well
101   as sterols (Alberts et al. 1992; Weldon et al. 1990).

102   Despite increasing interest in the chemical ecology of lizards, there are no such studies on
103   one of the best known reptile species in the world: the marine iguana (*Amblyrhynchus cristatus*).
104   This species is endemic to the Galápagos Islands - a world-renowned biodiversity hotspot
105   formed by 13 major islands (>10 km^2), 6 smaller ones and numerous islets in the eastern Pacific.
106   One of the most interesting aspects of the species' behavioural ecology is its lek-mating system.
107   A lek is defined as a mating system in which there is no parental care, males aggregate in
108   clusters, and females benefit from no resources other than the gametes available in the male
109   territories (Höglund & Alatalo 1995). Lek-mating behaviour has not been studied in detail for
110   most of reptiles. In contrast, marine iguana lekking has received considerable attention over the
111   past decades, and the system is reasonably well described. At the beginning of the mating season,
112   male iguanas establish small clustered territories along the rocky coastline. These territorial
113   males display head bobbing behaviour in order to attract females to their territories, presumably
114   to mate and also to intimidate competing males that are approaching the territory of the dominant
115   male (Wikelski et al. 1996). However, males that cannot establish territories (marginal males)
116   have far less reproductive success than territorial ones, and basically rely on forced copulations
117   with females that remain outside of the lek territories (Partecke et al. 2002; Wikelski et al. 1996).
118   Apparently, females may benefit from mating within specific males´ territories, as the territorial
119   male protects them from harassment by others (Trillmich & Trillmich 1984; Wikelski et al.
120   1996). The importance of chemical signals in lekking species has been verified in insects. The

121 mosquito *Aedes aegypti* uses an aggregation-pheromone to mediate lek establishment (Cabrera &

122 Jaffe 2007) and males of the sand fly *Lutzomyia longipalpis* may use pheromones to attract

123 females to their leks (Jones & Hamilton 1998). Therefore, the lek-mating system offers a

124 valuable natural setup to investigate questions regarding chemical signalling.

125 Several factors suggest that marine iguanas might rely heavily on pheromones for

126 intraspecific communication. Firstly, they possess femoral glands thatare the source of chemical

127 signals for many species of lizards (Mayerl et al. 2015). Secondly, observations have revealed a

128 "substrate-licking" behaviour in marine iguanas; (Carpenter 1966) observed that male iguanas

129 protrude their tongue onto the lava rocks as if testing for scent marks. Further, female iguanas

130 have been reported to lick the sand intensively when exploring new areas to lay eggs (Rauch

131 1988). Tongue licking is a chemoreceptive response to recognition of marked substrates in

132 iguanids (De Fazio et al. 1977; Krekorian 1989) and suggests that *A. cristatus* might use

133 conspecific olfactory cues for intraspecific communication.

134 Our study is the first to analyze the lipophilic fraction of femoral gland secretions in

135 Galápagos iguanas. By performing intensive sampling of all major island populations and

136 subspecies of marine iguanas (see (Miralles et al. 2017)), we seek to characterize the lipophilic

137 profile of femoral glands, as well as to explore the variation between populations originating

138 from distinct islands of the archipelago. In addition to sampling marine iguanas, we also

139 collected femoral secretions of land iguanas (*Conolophus subcristatus*). *Conolophus* is the sister

140 lineage of *Amblyrhynchus,* having diverged around 4.52 million years ago, most likely on the

141 Galápagos Archipelago (MacLeod et al. 2015). Therefore, we compare the chemical profile of

142 marine iguanas with their closest taxon, to investigate the level of differentiation in chemical

143 signals between the two. Marine iguanas have a great mobility and could even be passively

144 dispersed by oceanic currents (Carpenter 1966; Higgins 1978; Lanterbecq et al. 2010).

145 Therefore, we expect that chemical composition has a low variation among populations of

146 marine iguanas. In contrast, we hypothesize that land and marine iguanas diverge strongly in

147 their chemical signals because they occupy totally distinct habitats and there is no gene flow

148 among both species.

149 A further aim of this study was to understand the functions of femoral gland compounds

150 in marine iguanas. For this purpose, we carried out behavioural observations in one population of

151 *A. cristatus*. Morphological and behavioural data of territorial males were collected during the

152   breeding season and correlated with data on gland chemistry. Body size, as well as the body
153   condition index, of male marine iguanas were estimated as determinant factors for female mate
154   choice and male dominance hierarchy (Wikelski et al. 1996). Territorial males were regularly
155   monitored to estimate their head-bobbing rate, and the presence of females in their territories was
156   recorded. Head-bobbing is a behaviour typically displayed by territorial males to defend their
157   territories from other males, as well as to attract females during the breeding season (Wikelski et
158   al. 1996). Dominant males, i.e. those with high display levels (i.e. frequent head bobbing) and
159   who are accompanied by females, should release certain chemicals in their territories that inform
160   conspecifics of their status.

161

162   # METHODS AND MATERIALS

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164   **Femoral gland sampling**

165

166   During the months of December and January of the years 2014 - 2016 (a period coinciding with
167   the mating season of marine iguanas), an overall of 196 iguanas were captured in 11 islands of
168   the Galápagos archipelago in order to sample their femoral glands. Of those, a subsample of 134,
169   representing all major island populations and subspecies (see Table S1 and Fig. 1 for an
170   overview), were used to examine the variation of chemical profiles among populations.
171   However, due to uneven sampling effort, the number of obtained samples per population was
172   unequal. Therefore a subset of 103 samples, with even sample size for each population was
173   randomly selected and used in statistical analyses in order to avoid any effects of an unbalanced
174   design. The remaining samples (62) were taken from territorial males of a single population (i.e.
175   *A. cristatus mertensi* from La Lobería on San Cristóbal) and used to understand the function of
176   chemical signals in intraspecific communication. In this way, chemical composition of femoral
177   secretions could be analyzed in relation to individual morphology and behaviour (see below:
178   **Morphological dataset** and **Behavioural dataset**). The marine iguanas were captured using a
179   pole with a lasso, and their femoral secretions were obtained by gently squeezing the femoral
180   glands. Femoral secretions were then placed in a 2 ml glass vial with dichloromethane. All
181   samples were stored cold (in portable coolers at approximately $4 - 8$ °C) during the field work,

182   and subsequently transported to laboratories in Germany where they were stored at -80 ºC, until

183   chemical analysis were performed.

184   In addition, during January 2016, we also sampled the femoral glands of three individuals

185   of land iguana *C. subcristatus*, which are housed in a spacious outdoor enclosure at the Charles

186   Darwin Research Station on Santa Cruz Island.

187   The Galápagos National Park authority granted the research permission for this study

188   (permit numbers: PC-22-14, PC-08-15 and PC-09-16).

189

190   **Chemical analysis and lipid characterization**

191

192   The chemical analyses were performed by Gas Chromatography coupled with Mass

193   Spectrometry (GC-MS), and Gas chromatograph with a flame ionization detector (GC-FID). GC-

194   MS analyses of natural compounds were performed on a GC 7890A/MSD 5975C from Agilent.

195   Mass spectrometry was performed in an electron ionization mode (EI) with 70 eV. Fused-silica

196   capillary columns and HP-5MS (Agilent Technologies, 30 m, 0.25 mm i.D. 0.25 μm film

197   thickness) were used, with helium as the carrier gas. GC-FID was performed using a HP-5

198   column (Agilent Technologies, 30 m, 0.25 mm i.D. 0.25 μm film thickness) using hydrogen (H_2)

199   as the carrier gas with a flame ionization detection (FID) system.

200   The temperature program for the GC-MS was: 50-5-5-320 (50 °C starting temp., hold

201   time 5 min then 5 °C/min increase up to 320°C). For GC-FID with autosampler the temperature

202   program 125-5-5-320 (125 °C starting temp., hold time 5 min, the 5 °C/min increase up to

203   320°C) was used.

204   The samples collected in December 2014 and January 2015 were preserved in 500 μL

205   dichloromethane. Aliquots of 20 μL from each sample were taken for derivatizations, and 0.8 μL

206   of internal standard (tridecyl acetate) was added. The samples collected in December 2015 and

207   January 2016 were stored in 200 μL dichloromethane. Aliquots of 5 μL were taken for

208   derivatizations and 0.4 μL of internal standard (tridecyl acetate) was added, adding extra 20 μL

209   dichloromethane. The addition of the internal standard ensured that similar concentrations of the

210   samples were injected into the GC/MS system.

211   Derivatizations of the samples were performed using diazomethane (CH_2N_2), synthesized

212   as an etheric solution from Diazald following Black's procedure for converting carboxylic acids

213 into their corresponding methyl ester (Black 1983). Elucidation of the structure was performed

214 by mass spectral comparison with data bases, and comparison with reference compounds

215 identified 13 major compounds. Fatty acids were identified as their corresponding methyl esters

216 (Table 1). In contrast to marine iguanas, land iguana samples also feature aldehydes in addition

217 to carboxylic acids (Table 1). Besides the additional aldehydes, hexadecyl hexadecanoate and di-

218 (9-octadecenoyl)-glycerol were also found (Table 1). The position of the double bonds was

219 confirmed by dimethyl disulfide (DMDS)/I_2-derivatization of methylated natural samples

220 according to published procedures (Bruns et al. 2013; Buser et al. 1983).

221

222 **Morphological dataset**

223

224 Morphological data and femoral secretions were collected in a group of territorial males to

225 explore whether the chemical compounds might inform of certain morphological traits relevant

226 for mate choice and/or dominance status. For this part, we focused on a single population (i.e. *A.*

227 *cristatus mertensi* from La Lobería, San Cristóbal Island).

228 During December 2014 and January 2015, a total of 53 male marine iguanas were

229 measured for their snout-ventral length (SVL) using a metric-tape ($\pm$ 1 mm), and for head width

230 (distance across the jaw at the widest point) using a calliper ($\pm$ 0.1 mm). Body mass was

231 recorded using a portable field scale ($\pm$ 10 g). In addition, femoral gland secretions were also

232 taken from all captured iguanas.

233 We estimated the relative head size as the residuals of a linear regression of log-

234 transformed head size versus SVL. The body condition was calculated as the residual from a

235 linear regression between body mass and SVL (both log-transformed) (Krebs & Singleton 1993;

236 Wikelski & Romero 2003).

237

238 **Behavioural dataset**

239

240 In order to understand the role of chemical signals in marine iguana lek-systems, we performed

241 an observational study at the La Lobería colony in San Cristóbal during the period from

242 14/12/2015 until 06/01/2016.

243   The study consisted of two parts performed in parallel: 1) a count to estimate the number
244   of iguanas in the study area was conducted; 2) the behaviour of territorial males in selected leks
245   was recorded and their femoral glands were sampled.

246

247   **Marine iguana count**

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249   A daily count was performed to provide a rough overview of the number of marine iguanas in
250   our study site (in total 12 days). In each count, the observer walked along the coastline, counting
251   all iguanas visible within the range of a ~1.5 km transect. The marine iguanas were classified in
252   three categories on the basis of their external features: territorial males, female-sized individuals
253   and juveniles, following the classification characteristics of MacLeod (et al. 2016). Territorial
254   males have enlarged dorsal crests, large body size and conspicuous breeding coloration. Female-
255   sized individuals lack such features, with their mostly dark and uniform coloration that contrasts
256   with the colourful red and green patches of the males. However, female-mimicking males or
257   sneakers cannot be identified by external features (Wikelski et al. 1996) and therefore we were
258   not able to distinguish sneaker males from females and both were classified as "female-sized"
259   individuals. Juveniles can be easily distinguished by their smaller size (approximately less than
260   70 cm in total length). Average numbers and standard deviations (SD) are given for each
261   category in the results section. The marine iguana count performed in this study intended to
262   provide a general idea of the number of iguanas in our study area but population size for *A.*
263   *cristatus* in this island has been already estimated from a mark-recapture study (see (MacLeod et
264   al. 2016)).

265

266   **Monitoring of territorial males**

267

268   The behaviour of 9 territorial males was monitored in their lek-mating areas. For this, we
269   selected two different sites at the "La Lobería" breeding colony on San Cristóbal (subspecies *A.*
270   *c. mertensi*) and an additional site on the neighbouring island of Santa Cruz (*A. c. hassi*).
271   Territorial males from each site were photographed at the beginning to allow identification from
272   digital images, and marked with numbers on the flanks using non-permanent white paint to allow
273   individual recognition during the observations. Territorial males show strong site-fidelity and

274   occupy the same territorial areas during the entire reproductive season (Wikelski et al. 1996). We

275   made an overall of 55 observations on focal territorial males, each lasting between 15 and 60

276   minutes, during which we noted the number of head bobs in each observation period.

277   Observations were made between 8 am and 6 pm local time always on sunny days. The head

278   bobbing rate was then calculated by dividing the total number of head bobs recorded by the total

279   time of the observation in minutes. We also monitored the presence or absence of females in the

280   male territory (females were considered as 'present' when one or more females were within

281   approximately two body lengths of the focal male).

282

283   **Statistical analysis**

284

285   The proportions of the compounds were re-standardized by calculating the relative contribution

286   of each compound (peak area of a focal compound) with respect to the total peak area for all

287   substances. Statistical analyses were performed with the interface Rstudio in R software version

288   3.3.2 (R Development Core Team 2013), as well as with Statistica v8.0 (Statsoft Inc., Tulsa, OK,

289   USA).

290   A permutational multivariate ANOVA (PERMANOVA) was used to test for significant

291   differences in chemical profiles among the different islands. Multiple pairwise comparisons were

292   performed by correcting for multiple testing on the basis of the Benjamini-Hochberg (B-H)

293   method, which controls for false-discovery rate (Benjamini & Hochberg 1995). Additionally, we

294   plotted the data in two dimensions using Non-Metric Multidimensional Scaling (NMDS). We

295   used SIMPER (similarity percentage analysis) to assess which compounds contributed most to

296   differences among populations (package "vegan"; Oksanen et al. 2011). This analysis allows

297   identifying which compounds contribute more to the observed pattern of similarity.

298   Furthermore, the relationship between morphological and behavioural variables, and the

299   proportion of specific lipids was examined in marine iguanas. In order to explore the degree of

300   collinearity in chemical composition data, a Principal Component Analysis (PCA) was

301   performed on the 13 lipophilic compounds, including those from the individuals for which

302   morphological or behavioural data had been gathered. We used the PCA to reduce the

303   dimensionality of the data, and to detect co-linearity among the levels of the various compounds.

304   Therefore, to avoid including co-linearity among independent variables in subsequent model

305   development, only those compounds that correlated best with each PC factor (i.e. variables with
306   the highest loadings) were used, and the remaining compounds were discarded (following
307   (Dormann et al. 2013); see Table S2). Compounds that correlated strongly with each PC factor
308   were selected as independent variables for further modelling, these were: tetracosanoic acid,
309   hexadecanoic acid, 11-eicosenoic acid and 9-octadecenoic acid.

310   In order to test the relationship between morphology and proportion of the volatile
311   compounds in secretions, we built three linear models which considered body size (SVL), body
312   condition, and relative head size as dependent variables, with the proportion of selected
313   compounds as explanatory variables. The probability that the chemical profile of a given male
314   influenced whether or not he was accompanied by females was modelled using a mixed effects
315   logistic regression (binomial family, "glmer" function of package: "lme4"; (Bates et al. 2014)).
316   The identity of the male was considered as a random factor. The best model, including the best
317   subset of independent variables, was selected following the AIC corrected for small sample size
318   with the use of the "glmulti" package (Calcagno 2013). As some iguanas had no 11-eicosenoic
319   acid, we tested whether the presence of this chemical explained their head bobbing behaviour by
320   performing a non-parametric Mann-Whitney U-test.

321

RESULTS

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Diversity of lipids in femoral glands of Galápagos iguanas

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326   A total of 13 lipids from marine iguanas were identified and quantified (Table 1 and Fig. 2). We
327   characterized 10 carboxylic acids with chain lengths ranging between C_{16} and $C_{24,}$ as well as
328   three steroids. The most common compound in the femoral glands was cholesterol (31.14 %)
329   followed by hexadecanoic acid (22 %). C_{20}-unsaturated fatty acids, such as 11-eicosenoic acid
330   (0.35 %) and 13-eicosenoic acid (0.1 %), were the rarest compounds in marine iguana secretions.

331   A total of 20 volatile compounds were found in the femoral glands of land iguanas. Of
332   these compounds, 8 were found in *C. subcristatus* but not in *A. cristatus* (Table 1). The exclusive
333   compounds included saturated and unsaturated carboxylic acids, as well as aldehydes.
334   Interestingly, aldehydes were totally absent in marine iguana femoral glands, and thus were

335   specific to land iguanas. 13-Eicosenoic acid was absent in land iguanas, but present in some

336   populations of marine iguanas (Table 1, 2).

337   Lipophilic profiles varied greatly among island populations of marine iguanas (Table 2,

338   Fig. 3). All populations shared 8 compounds, these were: Hexadecanoic acid, 9-octadecenoic

339   acid, octadecanoic acid, 5, 8, 11, 14-eicosatetraenoic acid, eicosanoic acid, docosanoic acid,

340   tetracosanoic acid and cholesterol. Marine iguanas from some islands (e.g. Isabela, Santa Cruz,

341   San Cristóbal (La Lobería), Floreana and Santiago) had the full range of lipids, thus presenting a

342   high chemical diversity. However, other islands lacked one or more compounds (e.g. Española,

343   Marchena, Pinta, and Genovesa). This case was especially striking in Pinta and Genovesa, in

344   which the diversity of lipids was the lowest of the entire archipelago, with only 8 and 9 lipophilic

345   compounds, respectively. C_{20}-unsaturated fatty acids (i.e. 11- and 13- eicosenoic acids) were

346   most often absent in marine iguana populations (Table 2). Accordingly, the chemical

347   composition was dependent on population origin (PERMANOVA, Pseudo $F_{11,\ 91}$ = 7.57, P =

348   0.001, Fig 3). Pairwise comparisons revealed significant differences among lipids of some

349   populations (Table S3). Surprisingly, secretion chemistry differed between populations

350   inhabiting different islands, but belonging to the same genetic cluster (e.g. Fernandina-Isabela

351   and Española-Floreana, see Table S3 and Table S4). Conversely, some populations belonging to

352   different genetic clusters had similar chemical profiles (e.g. Pinta-Santa Fe and San Cristóbal-

353   Fernandina). In the case of the iguanas from Punta Pitt and La Lobería, which inhabit the same

354   island (San Cristóbal) but are genetically distinct, pairwise comparisons showed that their

355   chemical secretions are rather similar (corrected P = 0.21).

356

357   **Morphological and behavioural data**

358

359   The body size (SVL) of focal marine iguanas ranged from 35 cm to 54 cm, and body mass

360   ranged from 3 kg to 7.75 kg. The body condition index ranged from -0.40 (worst condition) to

361   0.26 (best condition). Male iguanas had a minimum head width of 3.6 cm and a maximum of 7.8

362   cm.

363   The average number of marine iguanas observed in the study area per study day was 135.

364   Female-sized were the most abundant group (average $\pm$ SD= 78 $\pm$ 8), followed by territorial

365   males (57 $\pm$ 8) and juveniles (10 $\pm$ 4). The head bobbing rate of the focal males ranged from 0.73

366 to 2.40 head bobs per minute. Some males were never accompanied by females, while others had

367 females in their territories during all the observations (raw behavioural data is shown in Table

368 S5).

369

370 **Functional correlates of femoral secretions with behaviour**

371

372 The PCA summarizes the variation of the 13 chemical compounds in four PCs, accounting for a

373 total of 75 % of the variation. The first PC (eigenvalue = 3.96, variance = 30.4 %) correlated best

374 with tetracosanoic acid (coefficient loading = 0.89). The second PC (2.39, 17.23 %) correlated

375 most with hexadecanoic acid (0.89). The third PC (1.98, 15.21 %) showed the highest correlation

376 with 11-eicosenoic acid (0.89). Finally, the fourth PC (1.6, 12.3 %) correlated with 9-

377 octadecenoic acid (0.78).

378 The best explanatory model for male body condition included two variables: 11-

379 eicosenoic acid and tetracosanoic acid (adjusted $R^2 = 0.24$, $F_{2, 46} = 8.41$, P < 0.001; 11-eicosenoic

380 acid: estimate = 16.18, t = 3.64, P < 0.001; tetracosanoic acid: estimate = 5.31, t = 2.32, P = 0.02;

381 Fig. 4). Body size (SVL) and relative head size were both independent of the proportions of the

382 chemicals found in the femoral glands (all P > 0.10).

383 The best model (i.e. lowest AICc, see Table 3) that predicted the probability of having

384 females in a male's territory included only one of the chemical compounds, i.e. 11-eicosenoic

385 acid. Males with higher proportions of 11-ecoisenoic acid were more likely to be accompanied

386 by females (estimate $\pm$ SE = 496.6 $\pm$ 166.5, Z = 2.98, P = 0.003; Fig. 5).

387 Furthermore, presence of 11-eicosenoic acid in femoral glands also correlated with an

388 increased head bobbing rate. Territorial males with 11-eicosenoic acid showed more head bobs

389 per minute (median = 1.84) than those lacking that compound (median = 1) (See Table S4).

390 However, this difference was only marginally significant (Mann Whitney U test: Z = -1.94, N =

391 7, P = 0.05, Fig. 6).

392

393 # DISCUSSION

394

395   Although many researchers have focused their attention on the ecology, behaviour and
396   microevolution of the Galápagos iguanas, studies on the chemical communication in these iconic
397   reptiles have so far been neglected. Our study is the first to survey the lipophilic compounds of
398   Galápagos iguanas, and to explore their possible function for intraspecific communication in *A.*
399   *cristatus*. Unlike many studies focusing on the chemistry of femoral gland secretions in lizards
400   (reviewed in (Weldon et al. 2008)), we surveyed not one but all major island populations of
401   marine iguanas, providing a complete overview of the intraspecific variation of lipophilic
402   substances in this species. In the following section, we discuss the general patterns of lipophilic
403   diversity between land and marine iguanas, as well across marine iguana populations.
404   Furthermore, we describe the composition of specific lipid compounds and their possible
405   functional correlates for chemical communication in the context of lek-mating.

406

407   **Diversity of femoral lipids in Galápagos iguanas**

408

409   The lipid profiles of marine and land iguanas were different. A total of 13 and 20 different lipids
410   were found in marine iguanas and land iguanas, respectively (Table 1). In detail, eight
411   compounds (fatty acids and aldehydes) were found in land iguanas but not in marine iguanas.
412   Most surprisingly, many of these compounds are aldehydes, a group of lipids that is completely
413   absent in marine iguanas, making these substances a characteristic difference between land and
414   marine iguanas. It is likely that land iguanas might have one or more enzymes that catalyze the
415   production of aldehydes. Given that land and marine iguanas diverged around 4.5 million years
416   ago on the Galápagos archipelago (MacLeod et al. 2015), enzymes catalyzing aldehyde synthesis
417   might have evolved independently in the land iguana lineage. Alternatively, genes for these
418   enzymes might have been lost in the marine iguana lineage, or are now down-regulated. A
419   transcriptome/genome analysis of both land and marine iguanas, as well as of closely related out-
420   group taxa, such as members of the genus *Ctenosaura* from Central America, could shed light on
421   these alternative explanations. Interestingly, aldehydes are present in the lizard *Psammodromus*
422   *algirus* (Martín & López 2006), but not in a phylogenetically close species, *P. hispanicus* (López
423   & Martín 2009). The presence of aldehydes in *P. algirus* could have a functional environmental
424   constraint, as this species inhabits grassy areas where substrate scent marking is difficult, and
425   therefore the presence of highly volatile substances like aldehydes would be more suitable for

426   short-range communication (Martín & López 2014). In the case of Galápagos iguanas, ecological

427   factors might also be possible drivers for the observed divergence in chemical signals between

428   both species. For example, in marine iguanas, marking rocky substrates could be essential for

429   establishing territories by dominant males. For this purpose, aldehydes may be poorly suited

430   since they volatilize too rapidly in the high humidity conditions, while carboxylic acids and

431   steroids would persist longer. A recent study on chemical signal functionality of terrestrial

432   vertebrates (amniotes) found that aldehydes are emitted from the sender's body rather than from

433   scent marks, likely due to their susceptibility to oxidation and degradation that would limit their

434   persistence in scent marks (Apps et al. 2015). Interestingly, aldehydes occurring in body odours

435   are much shorter than those found in land iguanas, confirming that aldehydes are part of femoral

436   gland secretions. However, further research should clarify their potential role in intraspecific

437   communication. Alternatively, differences in chemical secretions between land and marine

438   iguanas could be due to their distinct diets. Marine iguanas live in humid coastal areas, and

439   adults dive to feed on algae (Trillmich & Trillmich 1986). Conversely, land iguanas, are strictly

440   terrestrial, feeding mainly on *Opuntia* cactus and other terrestrial vegetation (Traveset et al.

441   2016). Therefore, it could be that interspecific differences in secretions are related to the

442   variation on feeding regimes between both species. Moreover, our results must be interpreted

443   carefully because land iguanas were kept in captivity and this could affect their diet as well.

444   Therefore, we cannot rule out that the differences on chemical secretions are plastic and a more

445   intensive sampling in natural populations should shed light on the divergence on gland chemistry

446   between both species.

447   In general terms, the femoral lipids present in *A. cristatus* are similar to those found in

448   other iguanids (Alberts et al. 1992), and are composed of ten carboxylic acids and three sterols.

449   The carboxylic acid chain lengths ranged between C_{16} and C_{24}. The most abundant compound

450   was cholesterol. This sterol is also one of the most common compounds found in the femoral

451   glands of many other lizard species (Martín & Lopez 2014). Cholesterol is thought to play an

452   important role in stabilizing other molecules, such as fatty acids found in gland secretions

453   (Escobar et al. 2003; Weldon et al. 2008). This would be especially important in hot habitats,

454   such as those occupied by the marine iguanas, where high temperatures may contribute to the

455   rapid degradation of chemical signals; therefore cholesterol could act as a protector of other

456   compounds with potential pheromone activity. However, the chemical composition of the

457   femoral glands varies greatly among populations of marine iguanas across the archipelago. For
458   example, cholesterol accounted for almost 40 % of the gland secretions in marine iguanas from
459   Isabela and Santa Cruz, but was less than 20 % of secretions from Pinta and Española iguanas.
460   Other compounds, such as 11- and 13-eicosenoic acids, were absent in some of the northern
461   islands (Marchena, Pinta, Genovesa), as well as in Santa Fe and northern San Cristóbal, but were
462   present in low proportions in the rest of the islands. Eicosenoic acids have been reported in green
463   iguanas but their relative amounts are much higher (1.4 %) than in marine iguanas (0.4 %). Such
464   differences in secretions of both species could arise to distinct ecological or microclimatic
465   conditions between both species, however, more research is needed to confirm this hypothesis.
466   Similarly, other studies on Mediterranean lizards showed high variation among island
467   populations within the same species. For instance, populations of the Balearic lizard *Podarcis*
468   *lilfordi* from different islets showed strong differences in the lipid composition of their femoral
469   secretions (Martín et al. 2013). Further, a study comparing gland chemistry of different
470   populations of the Skyros wall lizard (*Podarcis gaigeae*), found that islet populations diverged
471   considerably more than mainland populations (Runemark et al. 2011). However, contrary to the
472   latter study, which suggested that chemical profile variation reflects genetic differentiation
473   between populations, the observed variation in marine iguana lipid profiles does not correlate
474   with the underlying genetic population structure and ongoing differentiation/speciation
475   processes. Based on analysis of microsatellite loci, most islands of the archipelago harbour a
476   distinct genetic cluster of marine iguanas, with some exceptions where islands share a cluster
477   (MacLeod et al. 2015). This is the case for iguanas from Española and Floreana, as well as for
478   the populations from Isabela and Fernandina. In our study, the chemical signatures of the iguanas
479   from these islands differed statistically. Moreover, a remarkable peculiarity of the genetic
480   structure of marine iguanas is found in the island of San Cristóbal, where two genetically distinct
481   subspecies are in an ongoing speciation process within the same island (MacLeod et al. 2015;
482   Miralles et al. 2017). One of the most important mechanisms driving speciation through
483   chemical signals is a mutation in a receptor that switches the specificity for a given pheromone
484   compound (Leary et al. 2012). This process promotes the development of divergent pheromones,
485   thereby establishing pre-mating barriers and precluding gene flow between populations.
486   However, we found no evidence that the ongoing speciation process in the marine iguanas of San
487   Cristóbal is reflected in the variation of lipophilic compounds. The femoral secretions of the two

488 main colonies of the distinct genetic clusters, i.e. La Lobería and Punta Pitt, have similar

489 chemical profiles. We therefore conclude that the profiles of lipophilic substances do not

490 correlate with the genetic diversity of populations and therefore, lipophilic compounds might

491 play only a minor role in prezygotic isolation between marine iguana populations. However, the

492 role that different compounds have is still unknown. It could be that small amounts in specific

493 compounds could play a role in keeping both populations apart. For example, 11- and 13-

494 eicosenoic acids were present in very small amounts in La Lobería colony but not in Punta Pitt,

495 and it could contribute to maintain reproductive isolation between them. Alternatively, among-

496 population differences in femoral secretions could be related to distinct diets. It is known that

497 dietary lipids can result in concomitant alterations of the lipid composition of important tissues

498 (Simandle et al. 2001). Marine iguanas are specialized on feeding macrophytic algae (Trillmich

499 and Trillmich 1986; Wikelski et al. 1993) and it could possible that the different islands vary in

500 the amount of grazable algae leading to the observed difference in chemical composition of

501 femoral glands.

502 **Behavioural and functional correlates of lipophilic compounds**

503

504 We found that marine iguanas with better body condition had higher amounts of 11-eicosenoic

505 acid and tetracosanoic acid (see Fig. 4). Saturated and unsaturated fatty acids, such as 11-

506 eicosenoic acid and tetracosanoic acid, are part of the energetic dietary lipids and fat stores of

507 lizards (Geiser & Learmonth 1994; Simandle et al. 2001). Therefore, femoral secretions rich in

508 11-eicosenoic acid and tetracosanoic acid might reflect male fat reserves, suggesting that only

509 marine iguanas in an optimal body condition can afford to allocate these lipids to their femoral

510 glands. Further support for this hypothesis comes from the fact that the presence of females in

511 leks correlates with levels of 11-eicosenoic acid in the territorial male's secretions. Male iguanas

512 that had 11-eicosenoic acid in their secretions had a higher head bobbing rate when compared to

513 those that lacked this compound, though the effect was only marginal. Female marine iguanas

514 choose territorial males for mating on the basis of their head bobbing behaviour - the greater the

515 investment in the display, the higher the reproductive success of the males (Vitousek et al. 2008).

516 Thus, dominant males may also enhance their mating probability by releasing high amounts of

517 11-eicosenoic acid within their territories. Evidence to support this idea is provided by other

518 reptile studies which show that unsaturated fatty acids may function as sexual attractors. For

519   instance, male rock lizards *Iberolacerta cyreni* produce scent marks with more oleic acid to
520   attract females and thereby increase their mating opportunities (Martín & Lopez 2010). A similar
521   mechanism is likely to occur in the lek-mating system of marine iguanas, where scent marking
522   would be essential in order to establish male territories (Partecke et al. 2002; Wikelski et al.
523   1996). Therefore, the results of this study suggest the presence of an underlying mechanism,
524   whereby scent marks are produced by males to increase their mating opportunities and therefore
525   their reproductive success. In order to attract females to their own territories, males would
526   provide reliable information on their condition by the allocation of more 11-eicosenoic acid in
527   their femoral gland secretions. However, other compounds such as docosanoic acid and 13-
528   eicosenoic acid were strongly related to tetracosanoic acid and 11-eicosenoic acid respectively,
529   and thus, could be that these compounds are also involved in intraspecific communication.
530   Therefore, our results must be carefully interpreted because other compounds are also good
531   pheromone candidates. Docosanoic acid and especially 13-eicosenoic acid are compounds that
532   should be investigated in future studies to test whether they play role in marine iguana chemical
533   communication. Moreover, further experimental research is needed to understand how female
534   marine iguanas use the information contained in male gland secretions. An experiment testing
535   female response to different concentrations of 11-eicosenoic acids could elucidate whether such
536   compound is a pheromone involved in mate choice.

537

538   **CONCLUSIONS**

539

540   Evolutionary divergence between land and marine iguanas is reflected by a remarkable
541   divergence of lipophilic substances that might have altered in response to environmental
542   constraints and the different life histories of both species. Differences in composition of
543   lipophilic substances seem not to be of central importance for the maintenance of genetic
544   population structure, or for the ongoing differentiation and speciation processes in marine
545   iguanas. However, males might use certain compounds, such as fatty acids like 11-eicosenoic
546   and tetracosanoic acids to signal a good body condition and to successfully attract females for
547   lek-mating. The composition and type of lipophilic substances in the femoral secretions of
548   marine iguanas seem to be more influenced by habitat conditions than by genetic differentiation.

549

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699 **Table 1**

700 Percentage (Mean ± SD) of major compounds present in marine and land iguana secretions, with

701 the retention indices (RI) of methyl esters of the fatty acids and cholesterol derivatives. Retention

702 indices have been calculated using a method developed by Van den Dool and Kratz (1963).

703 Compounds specific for land iguanas have been given in bold (aldehydes are marked with an*).

704

Lipids (Mean ± SD)			
Marine iguana	Land iguana	Compound name	RI
-	2.59 ± 1.65	**Hexadecanal***	**1813**
-	0.55 ± 0.74	**7-Hexadecenoic acid**	**1904**
-	1.48 ± 1.08	**9-Hexadecenoic acid**	**1908**
22.41 ± 12.01	19.99 ± 12.19	Hexadecanoic acid	1928
-	0.48 ± 0.83	**9-Octadecenal***	1993
-	0.27 ± 0.47	**11-Octadecenal***	1997
-	0.59 ± 0.67	**Octadecanal***	2016
-	1.55 ± 0.37	**Di-(9-Octadecenoyl)-glycerol**	2473
13.68 ± 4.81	20.40 ± 12.34	9-Octadecenoic acid	2099
1.45 ± 1.25	4.72 ± 2.80	11-Octadecenoic acid	2104
12.12 ± 7.15	8.37 ± 4.21	Octadecanoic acid	2125
4.85 ± 2.81	1.92 ± 1.06	5,8,11,14-Eicosatetraenoic acid	2258
0.35 ± 1.01	0.26 ± 0.15	11-Eicosenoic acid	2299
0.1 ± 0.23	-	13-Eicosenoic acid	2306
3.43 ± 2.58	0.55 ± 0.31	Eicosanoic acid	2326
2.41 ± 1.75	0.47 ± 0.16	Docosanoic acid	2501
2.13 ± 1.66	0.55 ± 0.37	Tetracosanoic acid	2536
31.14 ± 13.14	29.67 ± 31.81	Cholesterol	3133
4.73 ± 4.46	0.11 ± 0.19	Cholestanol	3143
1.11 ± 1.39	1.32 ± 0.75	Cholestanone	3187
-	4.11 ± 5.3	**Hexadecyl hexadecanoate**	**3361**

705 **Table 2**

706 Percentage (Mean ± SD) of the lipophilic compounds indentified and quantified in the distinct

707 populations of marine iguanas (*A. cristatus*). Most influential compounds contributing to

708 dissimilarity among populations (SIMPER analysis) are marked in bold. *Abbreviations for the*

709 *populations: IS=Isabela, MAR=Marchena, PIN=Pinta, GEN=Genovesa, SAN=Santiago,*

710 *ESP=Española, FL=Floreana, SFE=Santa Fe, FDA=Fernandina, CRUZ=Santa Cruz, SRL=*

711 *San Cristóbal (La Lobería), SRPC= San Cristóbal (Punta Pitt).*

712

	IS	MAR	PIN	GEN	SAN	ESP	FL	SFE	FDA	CRUZ	SRL	SRPC
Hexadecanoic acid	13.17 ± 2.48	29.17 ± 15.15	38.47 ± 13.07	32.45 ± 16.63	29.68 ± 7.82	38.77 ± 9.79	11.51 ± 2.41	24.29 ± 8.72	18.91 ± 3.15	14.92 ± 2.95	15.84 ± 3.15	21.46 ± 12.49
9-Octadecenoic acid	14.61 ± 2.53	12.48 ± 6.61	8.83 ± 8.43	12.52 ± 5.99	13.08 ± 4.54	17.53 ± 4.50	12.07 ± 1.75	15.73 ± 3.03	13.68 ± 2.27	15.16 ± 2.10	11.05 ± 6.42	15.16 ± 5.90
11-Octadecenoic acid	**2.60 ± 0.65**	**0.61 ± 0.95**	**0.00 ± 0**	**0.00 ± 0**	**1.98 ± 1.89**	**0.32 ± 0.90**	**1.94 ± 0.68**	**0.70 ± 1.07**	**2.03 ± 0.79**	**2.05 ± 0.36**	**2.59 + 0.96**	**0.87 ± 0.94**
Octadecanoic acid	7.98 ± 1.42	17.42 ± 13.11	22.07 ± 13.69	16.24 ± 7.76	14.28 ± 4.04	19.80 ± 5.85	6.77 ± 1.08	10.61 ± 2.89	10.78 ± 1.88	8.32 ± 1.41	8.90 + 1.06	12.75 ± 8.91
5,8,11,14-Eicosatetraenoic acid	5.76 ± 1.01	4.49 ± 3.73	3.39 ± 4.55	2.10 ± 3.59	3.53 ± 2.52	4.47 ± 4.92	4.94 ± 0.81	3.28 ± 3.16	6.28 ± 1.02	6.04 ± 0.84	6.26 ± 1.21	5.23 ± 2.95
11-Eicosenoic acid	**1.00 ± 0.24**	**0.00 ± 0**	**0.00 ± 0**	**0.00 ± 0**	**0.12 ± 0.31**	**1.20 ± 3.39**	**0.39 ± 0.35**	**0.00 ± 0**	**0.08 ± 0.28**	**0.62 ± 0.45**	**0.51 + 0.38**	**0.00 ± 0**
13-Eicosenoic acid	**0.59 ± 0.24**	**0.00 ± 0**	**0.00 ± 0**	**0.00 ± 0**	**0.08 ± 0.22**	**0.00 ± 0**	**0.19 ± 0.27**	**0.00 ± 0**	**0.00 ± 0**	**0.19 ± 0.24**	**0.04 + 0.13**	**0.00 ± 0**
Eicosanoic acid	2.75 ± 0.57	3.68 ± 3.09	2.36 ± 3.30	1.58 ± 2.91	6.56 ± 2.09	6.23 ± 5.06	2.80 ± 1.52	1.35 ± 2.04	4.12 ± 1.05	3.11 ± 0.5	3.57 + 0.82	3.30 ± 2.11
Docosanoic acid	1.94 ± 0.34	2.39 ± 2.75	2.14 ± 2.95	0.48 ± 1.27	3.91 ± 2.12	1.94 ± 2.90	3.12 ± 0.58	1.85 ± 2.21	2.82 ± 0.80	2.29 ± 0.33	2.71 + 0.68	2.87 ± 1.87
Tetracosanoic acid	**1.81 ± 0.28**	**1.98 ± 2.27**	**0.87 ± 1.96**	**0.58 ± 1.55**	**4.02 ± 2.01**	**1.49 ± 2.80**	**2.76 ± 0.48**	**1.28 ± 1.92**	**2.57 ± 0.68**	**2.14 ± 0.36**	**2.91 + 0.88**	**2.35 ± 1.79**
Cholesterol	38.50 ± 5.68	23.54 ± 13.93	21.93 ± 14.11	32.59 ± 16.21	18.03 ± 6.01	8.23 ± 9.22	40.41 ± 5.12	34.49 ± 6.28	33.18 ± 6.08	39.56 ± 6.61	37.65 ± 8.17	29.98 ± 16.25
Cholestanol	7.85 ± 1.93	3.86 ± 6.30	0.00 ± 0	1.42 ± 3.7	4.56 ± 6.48	0.00 ± 0	10.53 ± 1.96	6.20 ± 5.97	4.32 ± 3.76	4.01 ± 2.08	5.45 ± 2.60	4.06 ± 2.56
Cholestanone	**1.41 ± 0.58**	**0.36 ± 0.89**	**0.00 ± 0**	**0.00 ± 0**	**0.15 ± 0.39**	**0.00 ± 0**	**2.54 ± 1.02**	**0.34 ± 1.03**	**1.21 ± 1.06**	**1.56 ± 0.74**	**2.51 ± 1.31**	**1.96 ± 2.41**

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714

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716

717   **Table 3**

718   Top five ranked models that examined the probability that presence of females in a specific

719   male's territory depends on the chemical composition of its femoral secretions. AICc=AIC

720   corrected for small sample sizes; ER=Evidence Ratio. The best model is shown in bold.

721

722

Probability of having females in male's own territory				
Variables included in the model	*AICc*	*Delta*	*Weight*	*ER*
11-Eicosenoic acid	**36.54**	**0**	**0.36**	**1.00**
11-Eicosenoic acid + Tetracosanoic acid	37.82	1.28	0.19	1.90
11-Eicosenoic acid + 9-Octadecenoic acid	38.36	0.54	0.14	1.31
11-Eicosenoic acid + Hexadecanoic acid	38.94	0.57	0.11	1.33
11-Eicosenoic acid + Tetracosanoic acid + Hexadecanoic acid	39.93	1	0.06	1.64

724 Figure caption:

725

726 **Fig. 1**

727 Map of the Galápagos Archipelago, showing the total number of femoral gland samples collected

728 per island, and the number of samples considered for statistical analysis in brackets. Island

729 abbreviations: GEN, Genovesa; MAR, Marchena; PIN, Pinta; SAN, Santiago; CRUZ, Santa

730 Cruz; SRL, San Cristóbal (La Lobería); SRPC, San Cristóbal (Punta Pitt); SFE, Santa Fe; ESP,

731 Española; FL, Floreana; IS, Isabela; FDA, Fernandina.

732

733 Fig. 2

734 Example of a chromatogram of marine iguana (SRL population). Each number correspond with

735 one of the identified compounds. 1= Hexadecanoic acid; 2 = 9-Octadecenoic acid; 3= 11-

736 Octadecenoic acid; 4 = Octadecanoic acid; 5 = 5,8,11,14-Eicosatetraenoic acid; 6 = 11-

737 Eicosenoic acid; 7 = 13-Eicosenoic acid; 8 = Eicosanoic acid; 9 = Docosanoic acid, 10 =

738 Tetracosanoic acid; 11 = Cholesterol; 12= Cholestanol; 13 = Cholestanone.

739

740 **Fig. 3**

741 a) Amount (percentage) of each chemical compound for all the sampled populations of marine

742 iguanas. Each colour represents one chemical compound.

743 b) Chemical composition plot, showing two-dimensional non-metric Multidimensional Scaling

744 (nMDS), which is based on the Bray-Curtis Similarity indices as calculated with *metaMDS* (in R;

745 using the *vegan* package). The closer the data point, the more similar the compound. Ellipses

746 represent CI=95%.

747

748 **Fig. 4**

749 Plot showing the relation between male body condition and the amount of a) 11-eicosenoic acid

750 and b) tetracosanoic acid. In grey 95 % CI.

751

752 **Fig. 5**

753 Plot showing the probability of the presence of females in male territories compared to the

754 amount of 11-eicosenoic acid. In grey 95 % CI.

755

756 **Fig. 6**

757 Box plot showing the head bobbing rate (Median $\pm$ Q1 and Q3) as a function of the presence or

758 absence of 11-eicosenoic acid.

760   **Fig. 1**

761

763   Fig. 2
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765
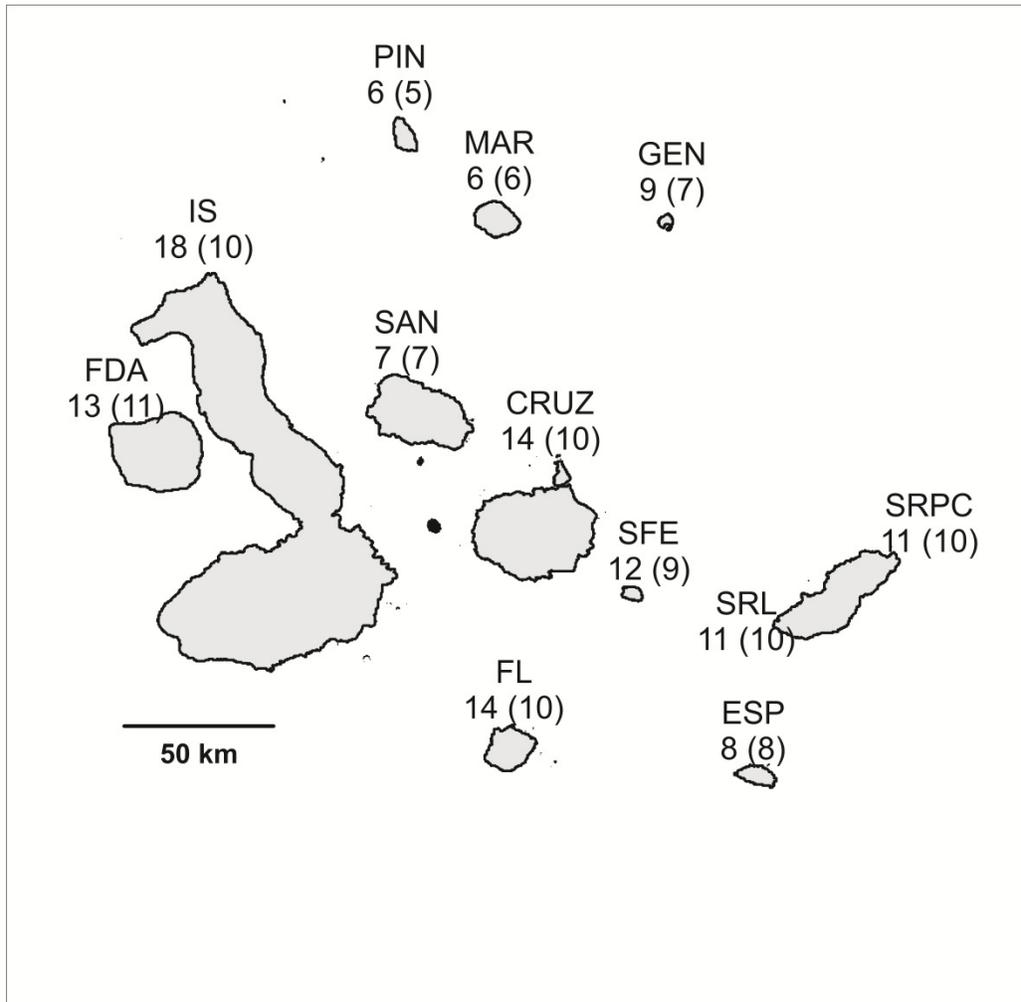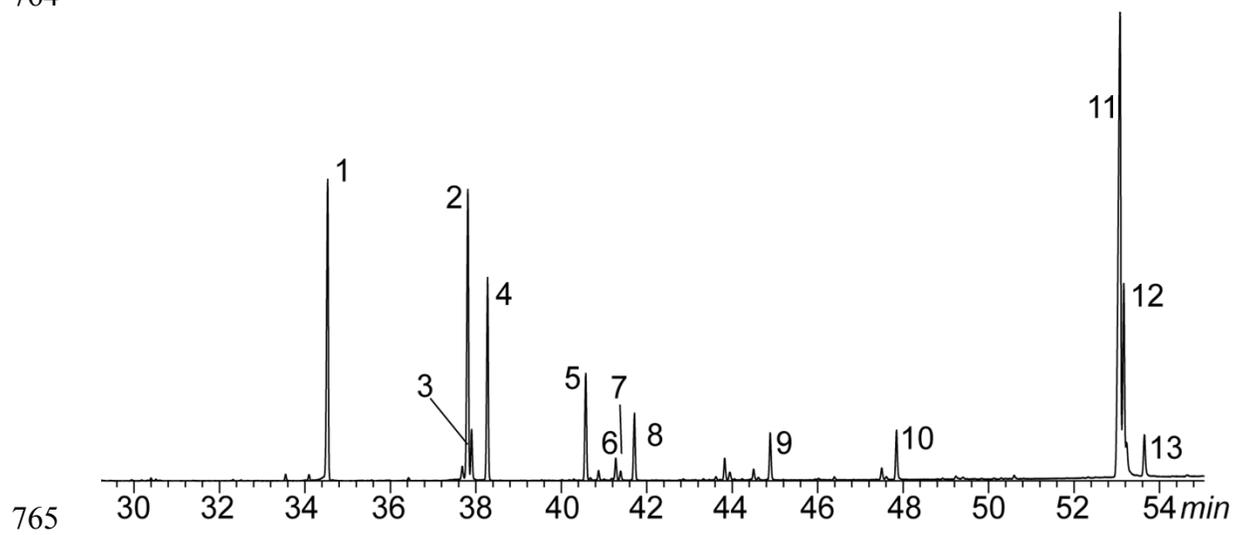
766   Fig. 3
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768   a)
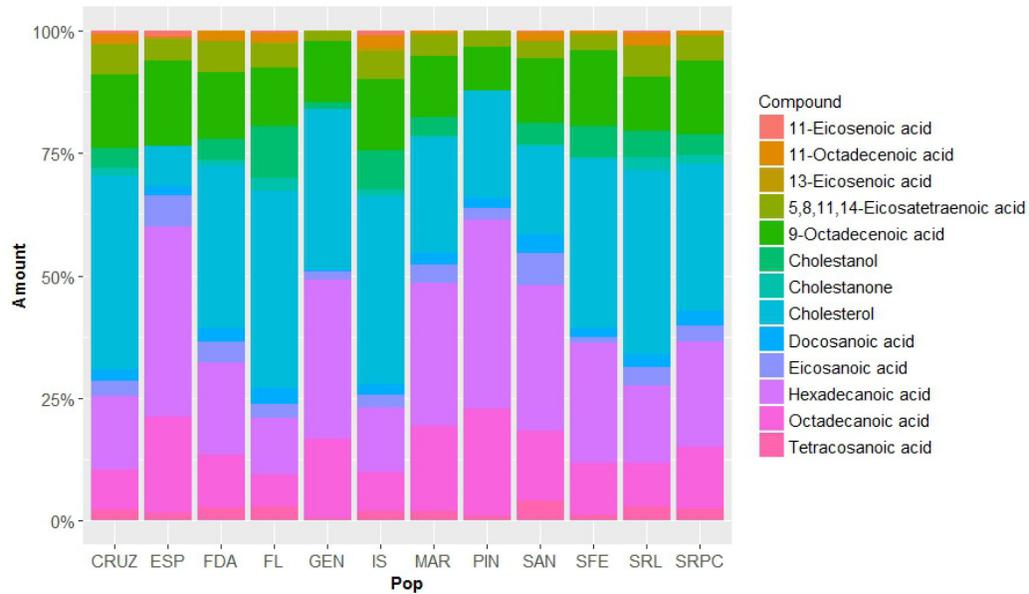
769
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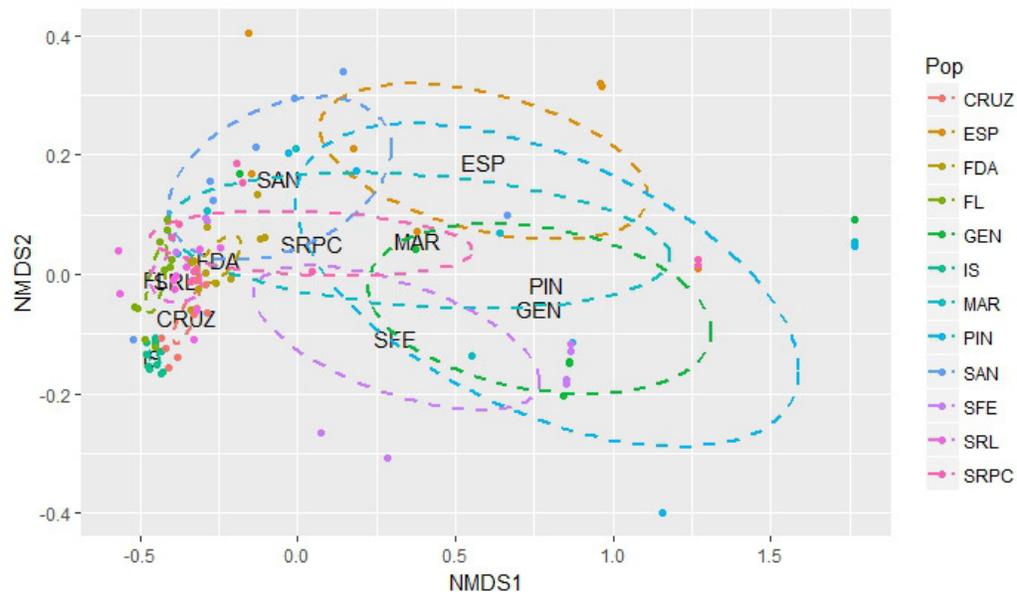
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774   **Fig. 4**

775   a)
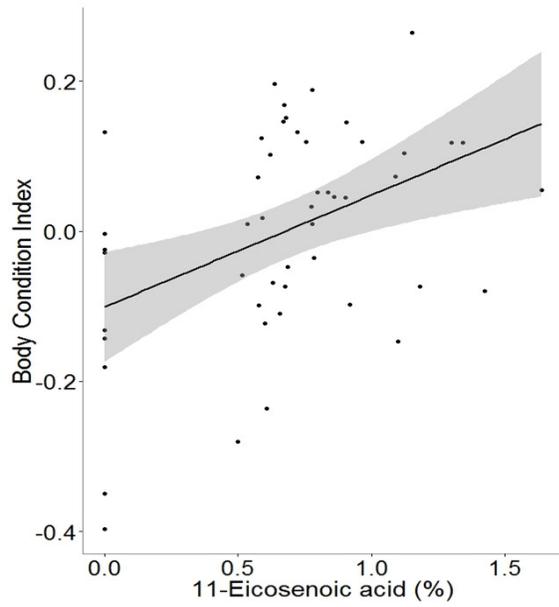
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777   **b)**
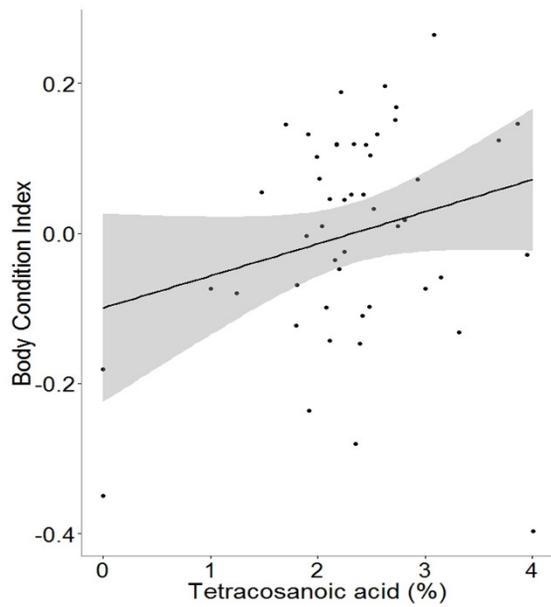
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780   **Fig. 5**
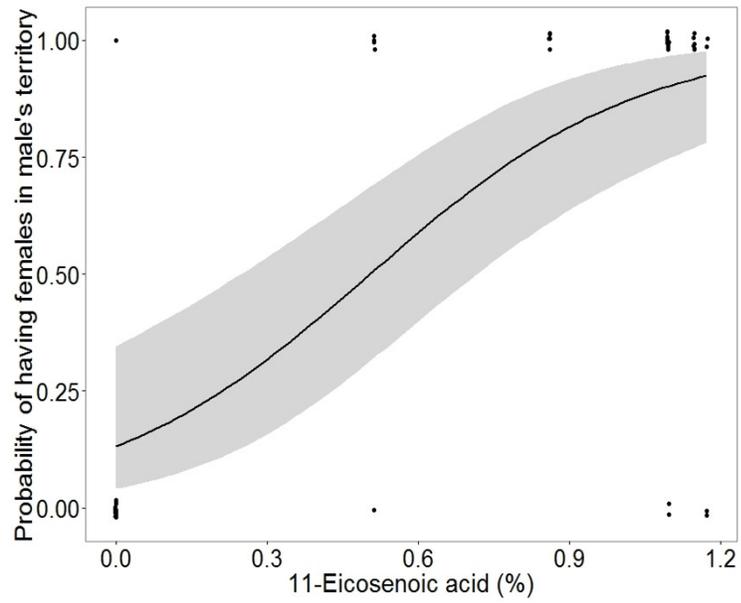
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783 **Fig. 6**

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