1 # Male sex pheromone components in *Heliconius* butterflies released
2 # by the androconia affect female choice

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16 **Abstract**

17 Sex specific pheromones are known to play an important role in butterfly courtship, and may

18 influence both individual reproductive success and reproductive isolation between species.

19 Extensive ecological, behavioural and genetic studies of *Heliconius* butterflies have made a

20 substantial contribution to our understanding of speciation. Male pheromones, although long

21 suspected to play an important role, have received relatively little attention in this genus. Here,

22 we combine morphological, chemical, and behavioural analyses of male pheromones in the

23 Neotropical butterfly *Heliconius melpomene.* First, we identify putative androconia that are

24 specialized brush-like scales that lie within the shiny grey region of the male hindwing. We then

25 describe putative male sex pheromone compounds, which are largely confined to the androconial

26 region of the hindwing of mature males, but are absent in immature males and females. Finally,

27 behavioural choice experiments reveal that females of *H. melpomene*, *H. erato* and *H. timareta*

28 strongly discriminate against conspecific males which have their androconial region

29 experimentally blocked. As well as demonstrating the importance of chemical signalling for

30 female mate choice in *Heliconius* butterflies, the results describe structures involved in release of

31 the pheromone and a list of potential male sex pheromone compounds.

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33 Key words: *Heliconius*, pheromone, sexual selection, mate choice, androconia, Lepidoptera,

34 reproductive isolation.

35

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Introduction

37
38   Sex pheromones are species-specific blends of chemical compounds that mediate

39   intraspecific communication between males and females (Wyatt, 2003, 2014). Among insects,

40   pheromone communication can involve a single chemical, but often relies on a complex

41   combination of multiple chemical components (Grillet, Dartevelle & Ferveur, 2006; Nieberding

42   et al., 2008; Symonds, Johnson & Elgar, 2012). This chemical complexity provides the potential

43   to convey sophisticated information, such as the inbreeding status of the emitter (Ando, Inomata

44   & Yamamoto, 2004; van Bergen et al., 2013; Menzel, Radke & Foitzik, 2016), mate quality

45   (Dussourd et al., 1991; Ruther et al., 2009), and species identity (Danci et al., 2006; Saveer et al.,

46   2014). Perhaps the best studied insect sex pheromones are those produced by female moths to

47   attract mating partners, often over long distances (Löfstedt, 1993; Smadja & Butlin, 2008).

48   However, male insects also produce sex pheromones (Eggert & Müller, 1997; Kock, Ruther &

49   Sauer, 2007; Ruther et al., 2009; Meinwald, Meinwald & Mazzocchi, 1969), and chemical

50   signalling can occur over short distances (Nishida et al., 1996; Mas & Jallon, 2005; Smadja &

51   Butlin, 2008; Wicker-Thomas, 2011; Grillet et al., 2012).

52   Sex pheromones can play a key role in determining the reproductive success of

53   individuals within a species, and may also result in reproductive isolation between species if

54   signals diverge (Johansson & Jones, 2007; Smadja & Butlin, 2008; Wyatt, 2014). Within

55   Lepidoptera, the importance of chemical signalling in mate choice and speciation is well

56   established among moth species (Phelan & Baker, 1987; Löfstedt, 1993; Bethenod et al., 2004;

57   Dopman, Robbins & Seaman, 2010; Lassance et al., 2010; Saveer et al., 2014). Most moths fly at

58   night, when visual signalling is unlikely to be as effective in attracting mates. In contrast,

59   butterflies are mostly diurnal and visual signals are usually important for initial mate attraction

60   (Vane-Wright & Boppré, 1993). However, chemical signals can play other roles in butterfly mate

61   choice, with evidence that close-range courtship interactions often involve pheromones emitted

62   by males, in contrast to the long-distance signalling with female-emitted pheromones more

63   commonly observed in moths (Vane-Wright & Boppré, 1993). Acceptance behaviour in the

64   queen butterfly *Danaus berenice*, for example, is regulated by a dihydropyrrolizine alkaloid

65   released by the male during courtship (Brower & Jones, 1965; Meinwald, Meinwald &

66   Mazzocchi, 1969; Pliske & Eisner, 1969). Another danaine butterfly, *Idea leuconoe*, displays

67   brush-like structures, called 'hair-pencils', emitting a mixture of volatiles during courtship,

68   which when applied to dummy males elicits an acceptance posture in females (Nishida et al.,

69   1996). *Pieris rapae* and *P. brassicae* both use macrocyclic lactones as a pheromone to induce

70   acceptance in females (Yildizhan et al., 2009). Finally, in *Bicyclus anynana* males with reduced

71   amounts of male sex pheromone have decreased mating success, implying a direct involvement

72   in reproductive fitness (Nieberding et al., 2008, 2012).

73   Here we focus on the potential role of male pheromones in *Heliconius* butterflies.

74   *Heliconius* is a diverse Neotropical genus, which has been extensively studied in the context of

75   adaptation and speciation (Jiggins, 2008; Supple et al., 2014; Merrill et al., 2015). These

76   butterflies are well known for Müllerian mimicry, in which unrelated species converge on the

77   same warning signal to more efficiently advertise their unpalatability to predators. Closely

78   related *Heliconius* taxa, however, often differ in colour pattern and divergent selection acting on

79   warning patterns is believed to play an important role in speciation (Bates, 1862; Jiggins et al.,

80   2001; Merrill et al., 2011b).

81   Male *Heliconius* display conspicuous courtship behaviours likely because the availability

82   of receptive females in nature is limited. Female re-mating is a rare event in *Heliconius* (Walters

83   et al., 2012) and males must compete to find virgin females within a visually complex

84   environment (Merrill et al., 2015). In addition, males donate a nutrient-rich spermatophore

85   during mating (Boggs & Gilbert, 1979; Boggs, 1981) which, together with costs associated with

86   extended copulation, will select for discrimination against less suitable mates in both sexes.

87   Crane (Crane, 1955) demonstrated that a combination of colour (hue) and movement stimulates

88   courtship by *Heliconius* males. More recently, it has repeatedly been shown across multiple

89   *Heliconius* species that males are more attracted to their own warning pattern than that of closely

90   related taxa (Jiggins et al., 2001; Jiggins, Estrada & Rodrigues, 2004; Kronforst et al., 2006;

91   Melo et al., 2009; Muñoz et al., 2010; Merrill et al., 2011a,b; Merrill, Chia & Nadeau, 2014;

92   Finkbeiner, Briscoe & Reed, 2014; Sánchez et al., 2015).

93   In addition to colour pattern, male *Heliconius* also use chemical signals to locate and

94   determine the suitability of potential mates. This includes the use of green leaf volatiles during

95   mate searching. Six-carbon alcohols and acetates are released by host plants in larger amounts

96   after leaf tissue damage caused by caterpillars, which adult males of the pupal mating species *H.*

97   *charithonia* then use to find potential mates (Estrada & Gilbert, 2010). Once males find pupae

98   they also use chemical cues to determine sex (Estrada et al., 2010). Supporting a further role for

99   chemical signals, *Heliconius erato* males distinguish between wings dissected from conspecific

100   and local *H. melpomene* females that are virtually identical in wing pattern, but this effect

101   disappears after wings have been washed in hexane (Estrada & Jiggins, 2008).

102   As well as attraction, chemicals can also be involved in repulsion. Males are repelled by a

103   strong odour released by previously mated females (Gilbert, 1976). This 'anti-aphrodisiac' is

104   produced by males soon after eclosion and is then transferred during copulation (Schulz et al.,

105   2008). The abdominal glands of male *H. melpomene*, for example, contain a complex chemical

106   bouquet consisting of the volatile compound (*E*)-β-ocimene together with some trace

107   components and esters of common C_{16} – and C_{18} – fatty acids with alcohols, where β-ocimene

108   acts as the main antiaphrodisiac component (Schulz et al., 2008). This antiaphrodisiac effect

109   occurs in several *Heliconius* species, which show species-specific patterns of scent gland

110   constituents (Gilbert, 1976; Estrada et al., 2011).

111   Despite the focus on male mate choice, analysis of courtship in *Heliconius* has shown

112   that females can exhibit rejection behaviours, such as raising their abdomen and flattening their

113   wings (Mallet, 1986; Klein & Araújo, 2010). There are also a number of observations that

114   indicate a role for chemical recognition in mate choice. *Heliconius erato* males separate their

115   wings during courtship to reveal the silvery overlap region, suggested to be involved in the

116   distribution of pheromones. This behaviour, described as androconial exposition, occurs in every

117   courtship that results in mating, suggesting that pheromones influence the female response

118   (Klein & Araújo, 2010). Additionally, direct evidence that *Heliconius* females use chemical

119   signals to distinguish conspecific males comes from studies of the closely related species *H.*

120   *timareta* and *H. melpomene*, which share the same warning patterns in Peru (Mérot et al., 2015).

121   Males experimentally treated with abdominal scent glands and wing extracts of heterospecifics

122   show a reduced probability of mating. Chemical analysis of both abdominal glands and whole

123   wings provides evidence for qualitative and quantitative differences in the chemical signatures

124   between these closely related species (Mérot et al., 2015).

125   Here, we investigate the role of chemical signalling in female mate choice in *Heliconius*

126   at three levels. First, we investigate morphological structures potentially associated with

127   pheromone production. In butterflies, a variety of species-specific structures including brushes,

128   fans, and differentiated scales on wings, legs or abdomen are used to expose pheromones

129   produced in associated glands (Wyatt, 2003; Nieberding et al., 2008). In particular, male-specific

130   scent glands, termed androconia, are common across the Lepidoptera. In male *Heliconius*, a

131   patch of shiny grey scales is present on the overlapping region of the hind and forewing (Fig. 1).

132   The observed sexual dimorphism in this trait suggests that these are androconia, and may be

133   associated with a male sex pheromone (Emsley, 1963). Furthermore, earlier authors have

134   identified brush-like scales in this region that are the putative site for pheromone production and

135   emission (Müller, 1912; Barth, 1952). Here we investigate the structure of these scales using

136   scanning electron microscopy. Second, we complement recently published chemical analysis of

137   whole *H. melpomene* wings (Mérot et al., 2015) by dissecting wing regions to identify those

138   associated with the production of compounds and identify the potential male sex pheromone

139   compounds isolated from this region. Finally, we carry out mate choice experiments in *H.*

140   *melpomene rosina*, *H. melpomene malleti*, *H. timareta florencia* and *H. erato demophoon* to test

141   the importance of pheromones for female choice.

142   **Methods**

143   Individuals used for morphological and chemical analyses were from an outbred stock of

144   *Heliconius melpomene plesseni* and *Heliconius melpomene malleti* (sold as *H. m. aglaope*),

145   maintained at the University of Cambridge insectaries (Fig. 1A). These two races are from the

146   region of a hybrid zone in the eastern Andes of Ecuador, and showed considerable inter-racial

147   hybridization in the stocks, so are treated here as a single population and referred to as the

148   Ecuador samples. These stocks were established from individuals obtained from a commercial

149   breeder (Stratford-Upon-Avon Butterfly Farm, Swans Nest, Stratford-Upon-Avon, CV37 7LS,

150   UK: www.butterflyfarm.co.uk). Laboratory stocks were maintained on the larval food plants,

151   *Passiflora menispermifolia* and *P. biflora*. Adult butterflies were fed on ~10% sucrose solution

152   mixed with an amino acid supplement (Critical Care Formula[®,] Vetark Professional, Winchester,

153   UK). Further chemical and behavioural analysis were carried out on the mimetic but distantly

154   related *H. melpomene rosina* and *H. erato demophoon* reared at the Smithsonian Tropical

155   Research Institute (STRI) facilities in Gamboa, Panama, and are referred to as the Panama

156   samples. Both males and females of the Panama samples were from outbred stocks established

157   from wild individuals collected in Gamboa (9˚7.4' N, 79˚42.2' W, elevation 60 m) within the

158   nearby Soberania National Park, and San Lorenzo National Park (9°17'N, 79°58'W; elevation

159   130 m). Larvae were reared on *Passiflora williamsi* and *P. biflora*. Adult butterflies were

160   provided with ~20% sugar solution with *Psychotria sp., Gurania* sp., and *Psiguiria sp.* as pollen

161   sources. Finally, behavioural experiments were carried out in the mimetic and closely related

162   species *Heliconius melpomene malleti* and *H. timareta florencia* reared at the insectaries of

163   Universidad del Rosario (UR) in La Vega, Colombia. These stocks derived from wild caught

164   individuals from Sucre, Caqueta (01°48'12" N, 75°39'19"W, elevation 1200 m). Larvae were

165   reared on *Passiflora oerstedii* and adults were provided with *Psiguiria sp.* as pollen source and

166   ~20% sugar solution.

167

168   *Morphological analysis*

169   The detailed morphology of androconial scales was determined using a Field Emission

170   Scanning Electron Microscope. Three males and two females of *H. melpomene* from Ecuador

171   were used for this analysis. The androconial grey scale region was dissected out from both hind

172   and forewings and attached to aluminium stubs with carbon tabs and subsequently coated with

173   20nm of gold using a Quorum/Emitech sputter coater. The gold-coated androconia were then

174   viewed in an FEI XL30 FEGSEM operated at 5kV. Images were recorded digitally using XL30

175   software at 500x magnification.

176

177

178 *Characterization of potential male sex pheromone*

179   Wing tissue from ten males (five newly emerged and five 10-day old) and five females

180 (10-day old) from the Ecuador stock was collected between November 2011 and March 2012 for

181 chemical analysis. Wings were dissected into four parts: forewing androconia, hindwing

182 androconia, forewing non-androconia and hindwing non-androconia. The 'androconia' regions

183 corresponded to the grey-brown region (Fig. 1B and C), with non-androconia corresponding to

184 the remaining portion of the wing. In females, a region corresponding in size and extent to the

185 grey-brown region seen in males was dissected. The dissected sections were then immediately

186 placed in 200μl hexane or dichloromethane in 2 mL glass vials and allowed to soak for three

187 hours. Initial analysis showed no major differences in extracted chemicals between hexane and

188 dichloromethane extracts (data not shown). Therefore, the more polar dichloromethane was used

189 in later analyses. Due to a larger available stock of *H. melpomene rosina* for behavioural

190 experiments, androconial tissue was also then collected from 20 males and 11 females (both 10-

191 12 days old) in Panama between February and July 2016. The tissue was soaked in 200μl

192 dichloromethane in 2ml glass vials, with PTFE-coated caps, for one hour. The extraction time

193 was shortened as this had no influence on the results (data not shown). The solvent was then

194 transferred to new vials and stored at -20°C. Samples were evaporated under ambient conditions

195 at room temperature prior to analysis.

196   Mature male androconial extracts from the Ecuador stock were analysed by gas

197 chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard model 5975 mass-

198 selective detector connected to a Hewlett-Packard GC model 7890A, and equipped with a

199 Hewlett-Packard ALS 7683B autosampler. All other Ecuador extracts were analysed by

200 comparison to the male androconial results. Extracts from the Panama stock were analysed by

201 GC/MS using a Hewlett-Packard model 5977 mass-selective detector connected to a Hewlett-

202 Packard GC model 7890B, and equipped with a Hewlett-Packard ALS 7693 autosampler. HP-

203 5MS fused silica capillary columns (Agilent, 30 m × 0.25 mm, 0.25 µm) were used in both GCs.

204 In both cases, injection was performed in splitless mode (250°C injector temperature) with

205 helium as the carrier gas (constant flow of 1.2 ml/min). The temperature programme started at

206 50°C, was held for 5 min, and then rose at a rate of 5°C/min to 320°C, before being held at

207 320°C for 5 minutes. Components were identified by comparison of mass spectra and gas

208 chromatographic Kovats retention index with those of authentic reference samples and also by

209   analysis of mass spectra. The double bond positions of unsaturated compounds were determined

210   by derivatisation with dimethyl disulfide (Buser et al., 1983). To confirm chemical structures,

211   alcohols were synthesised from the corresponding methyl esters by reduction according to

212   established procedures (Becker & Beckert, 1993, p. 570). The aldehydes were synthesised by

213   oxidation of the respective alcohols (More & Finney, 2002).

214   Compounds found in the extracts were quantified using gas chromatography with flame

215   ionisation detection with a Hewlett-Packard GC model 7890A or 7890B equipped with a

216   Hewlett-Packard ALS 7683B (Ecuador) or 7693 (Panama) autosampler. A BPX-5 fused silica

217   capillary column (SGE, 25 m × 0.22 mm, 0.25 µm) was used in both cases. Injection was

218   performed in splitless mode (250°C injector temperature) with hydrogen as the carrier gas

219   (constant flow of 1.65 ml/min). The temperature programme started at 50°C, held for 5 min, and

220   then rose to 320°C with a heating rate of 5°C/min. Pentadecyl acetate (10.1 ng) or (Z)-4-

221   tridecenyl acetate (1 ng) were used as internal standard for Ecuador samples, and 2-

222   tetradecylacetate (200ng) for Panama samples. Only compounds eluting earlier than hexacosane

223   were considered for analysis. Later compounds were identified as cuticular hydrocarbons, 2,5-

224   dialkyltetrahydrofurans, cholesterol and artefacts (e.g. phthalates or adipates). The variability in

225   the late eluting cuticular hydrocarbons was low and did not show characteristic differences

226   between samples.

227   For the Ecuador samples, groups were visualised as boxplots, due to the high frequency

228   of absent compounds in the samples. We then used non-parametric Kruskal-Wallis to test for

229   differences between the amounts of compounds present in different wing regions of mature

230   males, and also between age and sex categories. This was followed up by Dunn post-hoc testing

231   (Dinno, 2017; Ogle, 2017), with Bonferroni correction.

232   For the Panama samples, due to the higher sample size, and larger number of compounds

233   identified we visualised the males and females as two groups using a non-metric

234   multidimensional scaling (NMDS ordination, based on a Bray-curtis similarity matrix. We used

235   the metaMDS function in the package vegan (Oksanen et al., 2017), with visualisation using the

236   package ade4 (Dray & Dufour, 2007). This was followed up with ANOSIM to compare

237   differences between groups, and non-parametric Kruskal-Wallis tests to determine which

238   compounds differed between sexes.

239

240   *Behavioural experiments*

241   To test female acceptance of male pheromones, behavioural experiments were conducted

242   in insectaries at STRI, Gamboa, Panama between February and July 2016, and also in insectaries

243   at UR in La Vega, Colombia between November 2015 and June 2016. One day old virgin

244   females were presented with a control male and a 'pheromone blocked' male, both of which

245   were at least ten days old. Males from Panama were treated with transparent nail varnish (Revlon

246   Liquid Quick Dry containing cyclomethicone, isopropyl alcohol, ethylhexyl palmitate, mineral

247   oil and fragrance) applied to wings, following Constanzo and Monteiro (Costanzo & Monteiro,

248   2007). Males from Colombia were treated with transparent nail varnish (Vogue Fantastic).

249   Pheromone blocked males had the dorsal side of their hindwing androconia blocked, whilst

250   control males had the same region on the ventral side of the wing blocked.

251   Males were randomly marked using a black Sharpie marker with an 'x' on either their left

252   or right wing for identification purposes during the experiment. In Panama, experiments began at

253   8.30am and males were left in the cage until 3pm. During mating, *Heliconius* pairs invariably

254   remain connected for at least an hour and so observations were made every hour to check for

255   matings. If no mating occurred on the first day, this was repeated the next day with the same

256   butterflies. Behavioural observations were recorded for 17 trials with *H. erato demophoon* and

257   31 trials with *H. melpomene rosina* for the first two hours of the experiment on day one.

258   Observations were divided into one minute intervals, during which both female and male

259   behaviours were recorded. In Colombia, experiments were conducted from 7am to 1pm,

260   checking every 30 minutes for matings. As before, if no mating occurred on the first day, the

261   experiment was repeated the next day with the same butterflies. Female behavioural observations

262   were recorded for 17 trials with *H. timareta florencia* and 18 trials with *H. melpomene malleti*

263   for the first two hours of the experiment on day one. Observations were divided into one minute

264   intervals and were recorded only when a male was actively courting the female. Four female

265   rejection behaviours were recorded: 'Flutter' refers to when a female has her abdomen raised and

266   quickly flaps her wings; 'Wings open' refers to when the female is alighted with wings open and

267   abdomen raised but without wing fluttering; 'Abdomen up' refers to when the female is alighted

268   with wings closed and abdomen concealed within the wings; 'Fly away' refers to when the

269   female flies away from the male. Male courtships, defined as hovering over the female, were

270   recorded. Mating outcome results were analysed with binomial tests. We used generalized linear

271   mixed models (GLMMs) with a binomial error distribution and logit link function to test whether

272   females respond differently to control and experimental males. The response variable was

273   derived from trial minutes in which males courted where females performed a particular

274   behaviour ('success') or did not ('failure'). Significance was determined with likelihood ratio

275   tests comparing models with and without male type included as an explanatory variable.

276   Individual female was included as a random effect in all models to avoid pseudoreplication. All

277   statistical analyses were performed with *R* version 3.3.1 (R Core Team, 2016), along with the

278   packages ggplot2 (Wickham, 2009), car (Fox & Weisberg, 2011) and binom (Dorai-Raj, 2014).

279

280

Results

Morphological analysis

We identified a marked sexual dimorphism in scale structure (Fig. 2). In the central region of the male hindwing androconia along vein $Sc+R_1$ we identified specialised scales (Fig. 2A), which were absent in females and in the forewing androconia of males (Fig. 2B, 2C). These scales had brush-like structures at their distal end (Fig. 2D), and were not detected in any other wing region examined. The brush-like scales were found in alternating rows with scales with a normal structure. Moving away from the $Sc+R_1$ wing vein, the density and width of these scales decreased, with isolated brush-like scales found completely surrounded by normal scales. In addition, the base of these brush-like scales was more swollen and glandular as compared to other scales (Fig. 3).

Characterization of potential male sex pheromone

We initially investigated candidate wing pheromone composition using a stock of butterflies from Ecuador. By use of GC/MS and synthesis, six compounds were consistently found in the male wing extracts from these samples (Fig. 4) that were identified as the aldehydes (*Z*)-9-octadecenal, octadecanal, (*Z*)-11-icosenal, icosanal, and (*Z*)-13-docosenal and the alkane henicosane (C_{21}).

Firstly, a comparison of different wing regions of 10-day old males was carried out (Fig. 5A). Henicosane was found in all regions of the wing and was not considered in further analysis. The amount of (*Z*)-9-octadecenal was not significantly different between area categories. Octadecanal, (*Z*)-11-icosenal, icosanal and (*Z*)-13-docosenal showed significant differences between wing areas. Post-hoc testing found that these four compounds were significantly more abundant in the hindwing androconia than the rest of the forewing and hindwing, but not the forewing androconia (see Table S1 for statistical details).

Secondly, the hindwing androconia of old males, old females, and young males were compared (Fig. 5B). With the exception of henicosane, the other compounds were observed to be age-specific and sex-specific. (*Z*)-9-octadecenal was found more in old males than young males or old females but this was not statistically significant. In contrast, octadecanal, (*Z*)-11-icosenal, icosanal and (*Z*)-13-docosenal showed significant differences between age and sex categories.

311   Post-hoc testing found that these compounds were all present in significantly greater amounts in

312   old males than young males or old females (See Table S2 for statistical details).

313   As stocks of *H. melpomene rosina* from Panama were available for behavioural assays,

314   we then investigated the chemical composition of this population, using a larger sample size.

315   These Panama samples showed some similarities to the Ecuadorean samples, although they

316   contained more compounds and in higher amounts (Fig. 4; Fig. S1 and Table S3). Females and

317   males grouped separately with NMDS visualisation, and these groups were significantly different

318   (Fig. S1). In this larger dataset, (*Z*)-9-octadecenal, octadecanal, (*Z*)-11-icosenal, icosanal, (*Z*)-13-

319   docosenal and henicosane were all found in significantly larger amounts in old males than old

320   females, along with many other compounds (Ta. S3). Small amounts of nonadecanal, methyl-

321   branched octadecanals and their respective alcohols occurred that had not been detected in the

322   Ecuador samples, potentially due to the difference in equipment sensitivity, genuine geographic

323   variation, or the fact that the Ecuadorean butterflies have spent more generations in captivity.

324   Additionally, syringaldehyde was present, which was not detected in the Ecuador samples.

325

326   *Behavioural experiments*

327   In our mate choice trials, females of all four species/races discriminated against

328   conspecific males in which pheromone transmission was experimentally blocked (Table 1).

329   Across all four taxa tested, only seven of 71 matings (9.8%) were with thepheromone blocked

330   male, with the remaining 64 matings (90%) being with the control (unblocked) males. This was

331   not due to altered male courtship attempts as control and experimental males courted equally in

332   three out of four species (Fig. S2). In experiments with *H. timareta florencia*, the control males

333   courted more than experimental males (Fig. S2).

334   We observed no consistent significant differences in female behavioural responses

335   towards control and experimental males (Fig. S3). Some female behaviours were observed more

336   often towards experimental males in experiments with *H. melpomene malleti* and *H. timareta*

337   *florencia* (Fig. S3). In particular, *H. melpomene malleti* females were more likely to open their

338   wings towards experimental males ($2\Delta\ln L=17.093$, d.f.=1, p<0.001), fly away ($2\Delta\ln L= 8.0356$,

339   d.f.=1, p<0.01) and also flutter ($2\Delta\ln L= 15.823$, d.f.=1, p<0.001). Similarly, *H. timareta*

340   *florencia* females were also more likely to open their wings towards experimental males ($2\Delta\ln L=$

341   22.909, d.f.=1, p<0.001), fly away ($2\Delta\ln L= 6.1368$, d.f.=1, p<0.001), and flutter ($2\Delta\ln L=$

342   26.037, d.f.=1, p<0.001). However, despite these differences, these behavioural responses were

343   not consistently observed across the four species/races tested. Some of these results are driven by

344   just a few individual females, with differences in behaviour no longer significant when they are

345   removed. Furthermore, although some significant female behavioural responses were seen for *H.*

346   *melpomene malleti* in Colombia, no corresponding difference was found for *H. melpomene*

347   *rosina* in Panama, where a larger number of courtships were observed, so caution should be

348   taken when interpreting these results.

349

350   **Discussion**

351   Visual cues are known to be important for mate finding and courtship behaviours by male

352   *Heliconius* butterflies, with implications for reproductive isolation and speciation (Merrill et al.,

353   2015). Here, we have shown that female choice based on chemical signalling is also important

354   for reproduction. We have identified compounds associated with sexually mature male wings

355   and described morphological structures putatively involved in pheromone release. Furthermore,

356   we have shown that chemical signalling is involved in mating in *Heliconius*, with females from

357   three different species across four races showing strong discrimination against males which have

358   had their androconia experimentally blocked.

359   Our results are broadly comparable with another recent analysis of wing compounds in

360   *Heliconius* (Mérot et al., 2015), although the previous study did not compare different wing

361   regions, or males and females of the same age. As the previous study also did not use synthesis

362   to identify compounds, our work is highly complementary and extends their results to confirm

363   region- and age-specific localization of compounds to older male androconia. Male *Heliconius*

364   do not become sexually active until several days after eclosion, so the absence of these

365   compounds from females and younger males is strongly suggestive of a role in mating

366   behaviour. The compounds are unlikely to be obtained from direct sequestration of compounds

367   from larval host plants as they are not present in young males or in older females. This suggests

368   that there could be genetic control of the production of these compounds, and so they have the

369   potential to play a role in reproductive isolation between species.

370   The restriction of these five putative male sex pheromones, (*Z*)-9-octadecenal,

371   octadecanal, (*Z*)-11-icosenal, icosanal, and (*Z*)-13-docosenal, to the hindwing androconia of

372   mature males (Fig. 5A) suggests that pheromone storage or production is restricted to the

373 hindwing. This is supported by the scanning electron microscope images which show special

374 brush-like scales in the androconial region (Fig. 2A), located primarily around and along the

375 hindwing vein Sc+R_1, similar to the depiction in Figure 73 of Emsley's previous morphological

376 analysis (Emsley, 1963). Similar scales have been described from light microscopy in other

377 *Heliconius* species, but not previously in *H. melpomene* (Müller, 1912; Barth, 1952). The base of

378 these special brush-like scales was more swollen and glandular as compared to other scales (Fig.

379 3), perhaps indicating a role in storage or production of pheromones by these scales. Trace

380 amounts of chemicals on the forewing androconia may be due to contact in the overlapping

381 portion of the fore- and hindwings, and both wings may play a role in dispersal of the

382 compounds during courtship.

383 Samples from Panama showed both a greater diversity and amounts of compounds (Fig.

384 S1 and Table S3). This might reflect an issue with inbreeding in the Ecuador population because

385 they were obtained from a commercial breeder, or technical differences between the two

386 locations where the analysis was performed. However, it could also reflect differences in rearing

387 conditions or genuine variation between geographic populations of *H. melpomene*. Further work

388 will be needed to confirm the nature and extent of geographic and individual variation in

389 pheromone composition.

390 Females exhibited a strong preference for males which did not have their androconia

391 blocked. This suggests that, as in other butterfly systems (Costanzo & Monteiro, 2007), female

392 *Heliconius* are actively involved in mating decisions. Nonetheless, there were no consistent

393 differences in the female behaviours we recorded in our experiments. It is possible that the

394 important female preference behaviours are subtle and were missed in our study. Alternatively,

395 female acceptance of a male may instead simply represent a decision to stop rejection

396 behaviours, and therefore not be associated with any particular characteristic behavioural

397 response.

398 It remains unclear which compounds are biologically active and exactly what information

399 is being conveyed. The signal clearly influences female mating decisions, and these compounds

400 may convey complex information about male species identity, quality, age etc. that are

401 interpreted by females. It is also unknown whether females, like males, use visual cues in

402 courtship. The use of multiple signals is common in animal communication (Candolin, 2003).

403 Whilst butterflies primarily use visual cues to locate mates (Kemp & Rutowski, 2011), it has

404   been shown in *B. anynana* that in addition to visual cues, chemical cues also play a role and are

405   equally important in sexual selection by female choice (Costanzo & Monteiro, 2007). Our work

406   establishes the potential for similar multimodal signalling in *Heliconius* butterflies.

407   This study provides evidence for the importance of pheromones in intraspecific mate

408   choice in *Heliconius* butterflies. Evolution of cues within populations could lead to reproductive

409   isolation between populations if both cues, and their corresponding preferences, diverge (Ptacek,

410   2000). In other butterflies, male wing compounds contribute to reproductive isolation between

411   closely related species (Grula, McChesney & Taylor, 1980; Phelan & Baker, 1987; Bacquet et

412   al., 2015). Evidence suggests that strong pre-mating barriers in addition to mate preference based

413   on colour wing pattern exist between *Heliconius cydno* and *H. melpomene* (that differ in colour

414   pattern) and between the latter and *H. timareta* (that are mimetic) (Mérot et al., 2017). For

415   example, *H. cydno* males show a preference for their own pattern over that of the closely related

416   *H. melpomene*, but will court wing pattern models of *H. melpomene*. *Heliconius cydno* males,

417   however, have virtually never been observed mating with *H. melpomene* females (Naisbit,

418   Jiggins & Mallet, 2001). On the other hand, although males of the mimetic *H. timareta florencia*

419   and *H. melpomene malleti* equally court female wing models of both species, interspecific

420   matings occur in very low frequency (Sánchez et al., 2015; Mérot et al., 2017). Furthermore,

421   male androconial compounds differ between species (Mérot et al., 2015; Mann et al.,

422   unpublished data), suggesting that they could play role in reproductive isolation. Future work

423   will allow us to explore the multidimensional aspect of speciation by understanding both male

424   and female choice, and the role that multiple modes of signalling could play in reproductive

425   isolation.

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685   Figure 1. A) *H. melpomene malleti* (Ecuador sample, left) and *H. melpomene plesseni* (Ecuador

686   sample, right). B) Dissected wings from specimens of *H. melpomene plesseni* showing sexual

687   dimorphism in the androconial region, with male (left) and female (right). For each sex, the left

688   set of wings shows the ventral surface and the right set the dorsal surface. C) Expanded view of

689   the male androconial region with arrow highlighting the vein $Sc+R_1$. The ventral side of the

690   forewing is on the top and the dorsal side of the hindwing is on the bottom. The pale grey-brown

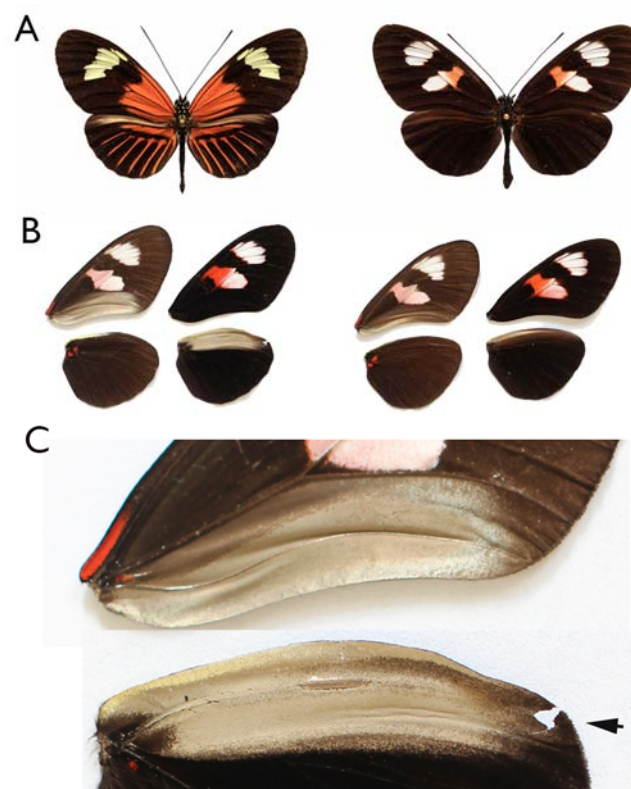691   region in the male wing was dissected for chemical analysis.

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698   Figure 2: SEM image of putative androconial scales of *H. melpomene*. Scales in the region of the

699   hindwing vein Sc+R$_1$ and forewing vein 1A are shown. A) male hindwing; B) male forewing

700   and; C) female hindwing at 500x magnification. d) Magnified view of brush-like structures of

701   the special scales in the male hindwing androconial region. Scale bars indicate 50 μm (A-C) and
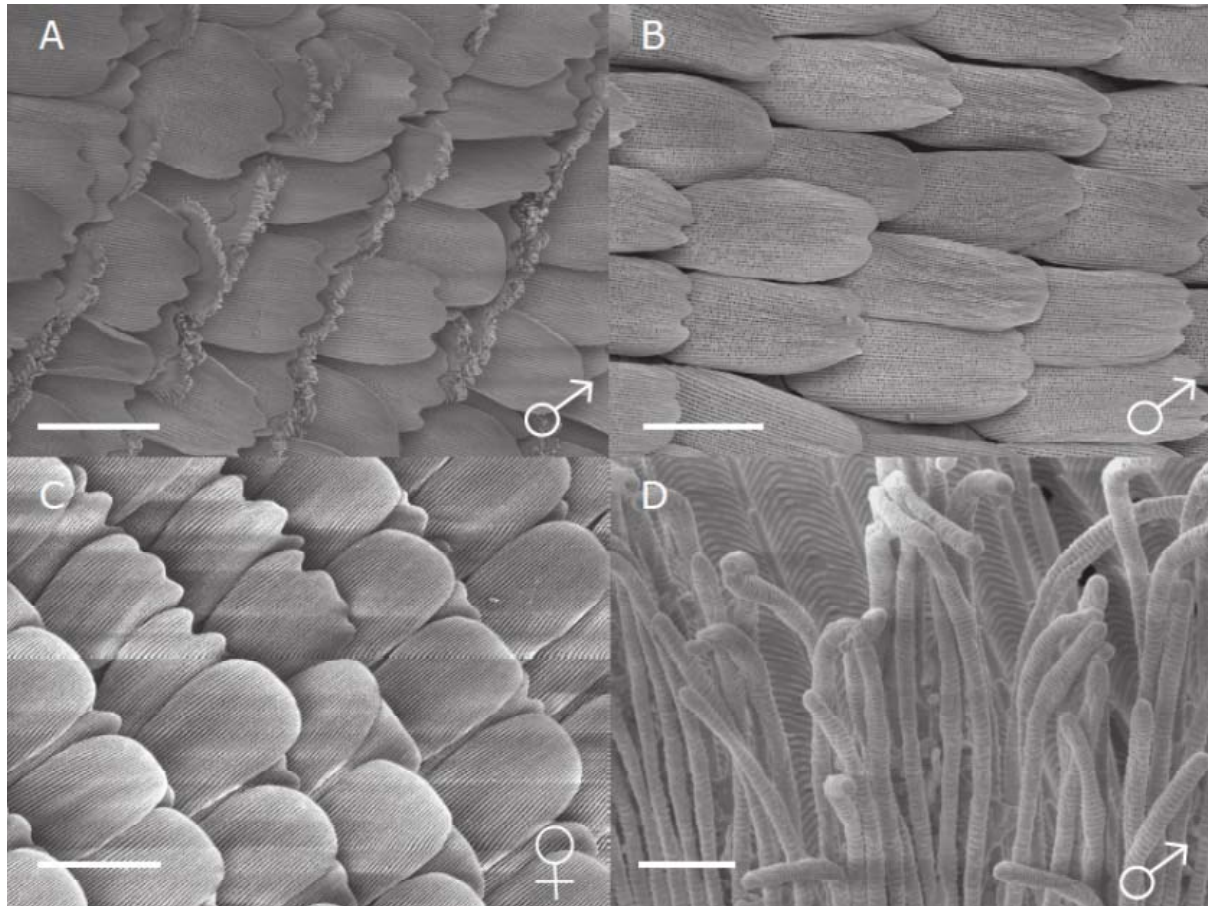
702   2 μm (D).

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705   Figure 3: SEM image of the base of a scale of hindwing androconia of *H. melpomene*. A) Scale

706   from androconial region of female. B) Scale from androconial region of male with brush-like

707   structures; the arrow highlights the bulge in the scale base in this region. Scale bars indicate 10
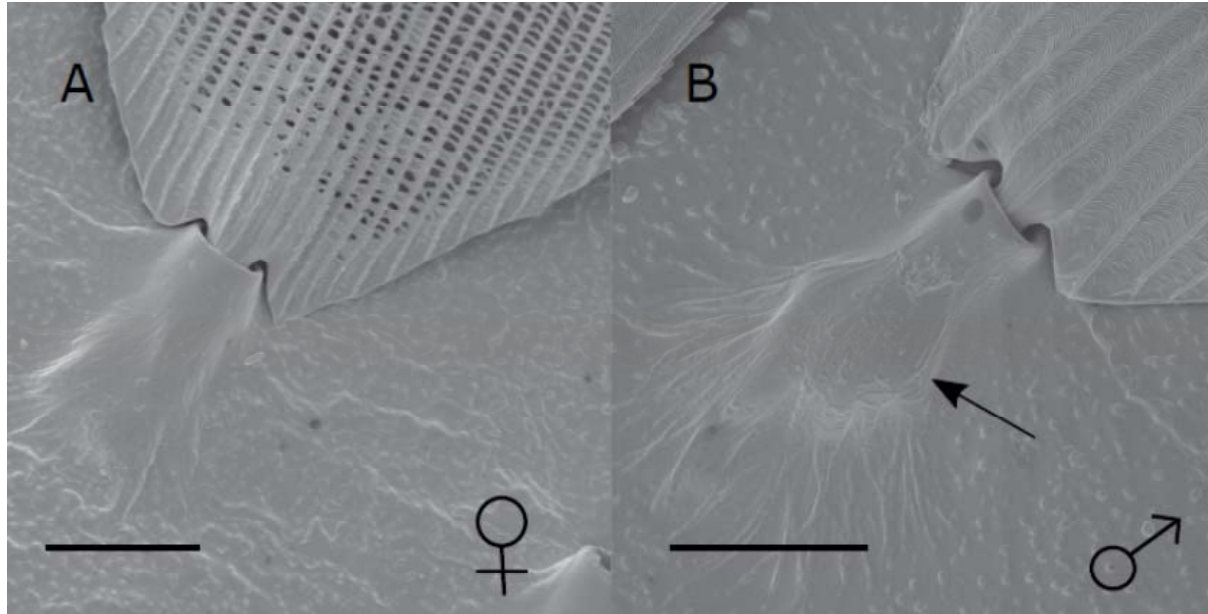
708   μm.

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726   Figure 4: Regional differences in male androconial extracts. Total ion chromatograms of

727   extracts from the androconial region of an Ecuadorian *H. melpomene* and a Panamanian *H.*

728   *melpomene rosina* male hindwing. 1: syringaldehyde; 2: (*Z*)-9-octadecenal; 3: octadecanal;

729   4: methyloctadecanals; 5: 1-octadecanol; 6: henicosane; 7: methyloctadecan-1-ols and

730   nonadecanal; 8: (*Z*)-11-icosenal; 9: icosanal; 10: (*Z*)-11-icosenol; 11: tricosane; 12: (*Z*)-13-

731   docosenal. All peaks eluting later than 44 min are cuticular compounds consisting of larger

732   *n*-alkanes, 2,5-dialkyltetrahydrofurans, cholesterol or are contaminations. IS, internal

733   standard.
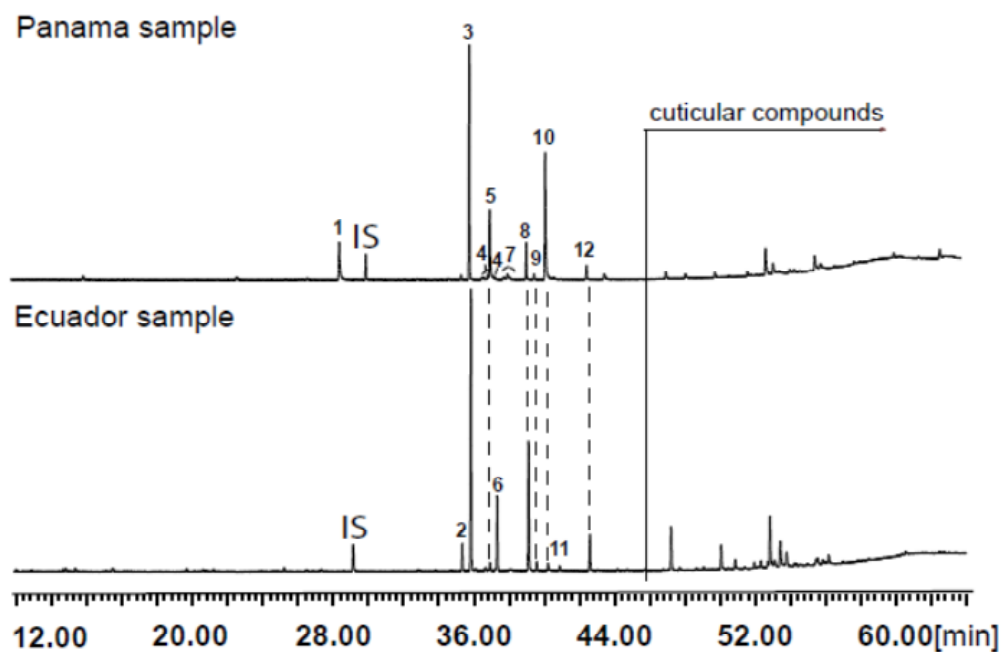

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737 Figure 5: Compounds detected by GC/MS of *H. melpomene* (Ecuador samples) wing extracts. A)

738 Presence of compounds in different wing regions of five males (10 days post-eclosion). B)

739 Presence of compounds in five females (10 days post-eclosion), five young males (0 days post-

740 eclosion) and five old males (10 days post-eclosion).
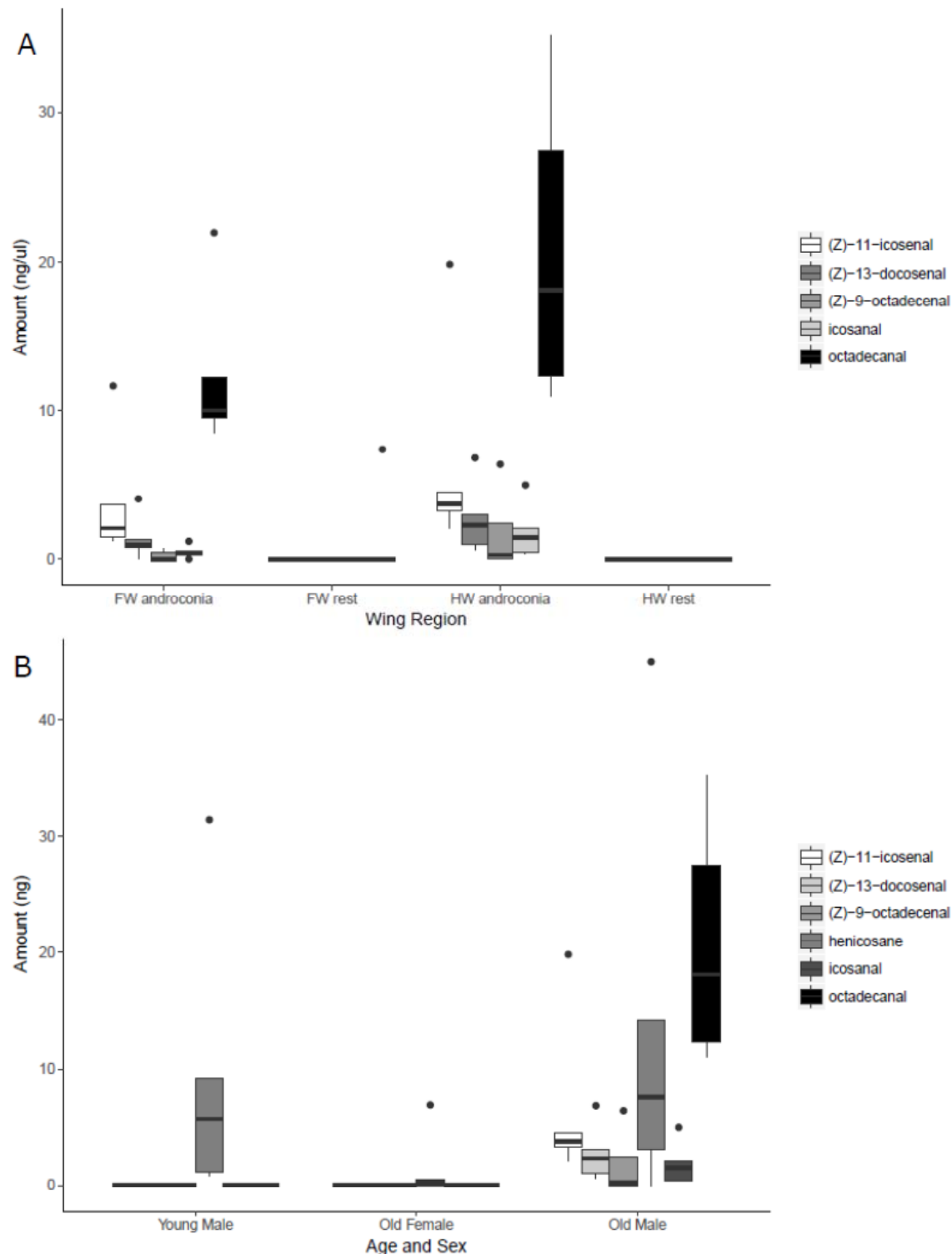

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Table 1: Outcome of mate choice trials across different species/races. Proportion of successful copulations with the control male was tested using an exact binomial test. Females mated significantly more with the control male than the experimental (pheromone-blocked) male in all four populations. Statistical analysis based on females which mated.

Species	Mated with control	Mated with experimental	Did not mate	p-value (Exact Binomial Test)
H. melpomene rosina	15	0	18	<0.001
H. erato demophoon	14	1	31	<0.001
H. melpomene malleti	19	3	8	<0.001
H. timareta florencia	16	3	5	<0.01