Male sex pheromone components in the butterfly *Heliconius melpomene*

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Abstract

Sex specific pheromones are known to play an important role in butterfly courtship, and may influence both individual reproductive success and reproductive isolation between species. Extensive ecological, behavioural and genetic studies of *Heliconius* butterflies have made a substantial contribution to our understanding of speciation. However, although long suspected to play an important role, chemical signals have received relatively little attention in this genus. Here, we combine morphological, chemical and behavioural analyses of a male pheromone in the neotropical butterfly *Heliconius melpomene.* First we identify specialized brush-like scales that are putative androconia, and lie within the shiny grey region found on the hindwing of males. We then describe six putative male sex pheromone compounds, which are largely confined to the androconial region of the hindwing of mature males, but not immature males or females. Finally, behavioural assays reveal subtle, but detectable, differences in female response to models scented with hindwing androconial extracts of mature conspecific males as compared to unscented controls. Collectively, the results describe structures involved in release of the pheromone and a list of potential male sex pheromone compounds triggering a behavioural response in females.

Key words: *Heliconius*, pheromone, androconia, Lepidoptera, sexual selection.

Introduction

Organisms use chemicals for a wide variety of forms of communication. In particular, sex pheromones are species-specific blends of chemical compounds mediating intraspecific communication between males and females, and can play key roles in both determining the reproductive success of individuals within a species, and in reproductive isolation between species (Wyatt, 2003, 2014). Some insects deploy single chemicals as signals, but in many insects, pheromone communication is dependent on complex combinations of chemical components (Grillet, Dartevelle, & Ferveur, 2006; Nieberding *et al.*, 2008; Symonds, Johnson, & Elgar, 2012). This chemical complexity provides the potential to convey sophisticated information (Nieberding *et al.*, 2012). The best studied insect sex pheromones are perhaps the chemicals produced by female moths to attract mating partners, often over long distances (Löfstedt, 1993; Smadja & Butlin, 2008). Male insects can also produce sex pheromones but these have generally received less attention (Wyatt, 2014).

Whilst variation in sex pheromone blend is now known to be a major determinant of reproductive isolation and speciation in many species of moths (Löfstedt, 1993; Smadja & Butlin, 2008; Lassance *et al.*, 2010), to date sex pheromones have been studied in just a few species of butterfly. Acceptance behaviour in the queen butterfly *Danaus berenice* is regulated by a dihydropyrrolizine alkaloid released by the male (Brower & Jones, 1965; Meinwald, Meinwald, & Mazzocchi, 1969; Pliske & Eisner, 1969). Another danaine butterfly, *Idea leuconoe* displays 'hair-pencils' during courtship, which contain a mixture of dihydropyrrolizine alkaloids, aromatics, terpenoids, hydrocarbons and a series of γ-lactones (Nishida *et al.*, 1996). This volatile mixture applied on dummy male butterflies elicits an acceptance posture in females. *Pieris rapae* and *Pieris brassicae* use macrocyclic lactones as a pheromone to induce acceptance in females (Yildizhan *et al.*, 2009). In *Bicyclus anynana* males with reduced amounts of male sex pheromone have decreased mating success implying a direct involvement in reproductive fitness (Nieberding *et al.*, 2008, 2012). Male wing compounds are also known to contribute to reproductive isolation between closely related species of butterflies (Grula, McChesney, & Taylor, 1980; Phelan & Baker, 1987; Bacquet *et al.*, 2015).

Here we focus on the potential role of a male contributed pheromone in *Heliconius* butterflies. *Heliconius* is a diverse neotropical genus, studies of which have contributed greatly to our understanding of speciation (Jiggins, 2008; Merrill *et al.*, 2015). These butterflies are known for Müllerian mimicry, where unrelated species converge on the same warning signal to more efficiently advertise their unpalatability to predators. However, closely related *Heliconius* taxa often differ greatly in colour pattern and divergent selection acting on warning patterns is believed to play an important role in speciation within the genus

(Bates, 1862; Jiggins *et al.*, 2001; Merrill *et al.*, 2011b). In particular, males are known to use colour pattern to recognize mates (Jiggins *et al.*, 2001; Jiggins, Estrada, & Rodrigues, 2004; Kronforst *et al.*, 2006; Melo *et al.*, 2009; Merrill *et al.*, 2011a; Merrill, Chia, & Nadeau, 2014; Finkbeiner, Briscoe, & Reed, 2014).

Some aspects of chemical signaling have been studied in *Heliconius*. For example, males are known to transfer an antiaphrodisiac pheromone to females during mating (Gilbert, 1976). Once mated, females produce a strong odor that acts to repel approaching males (Schulz *et al.*, 2008). Studies in *H. melpomene* have shown that abdominal glands of males contain a complex odor bouquet consisting of the volatile compound (*E*)-β-ocimene together with some trace components and esters of common C16 – and C18 – fatty acids with alcohols, where β-ocimene acts as the main antiaphrodisiac pheromone. This bouquet is formed during first few days of eclosion and transferred during copulation to the females (Schulz *et al.*, 2008). This antiaphrodisiac effect can be observed in several *Heliconius* species, which show species-specific patterns of scent gland constituents (Gilbert, 1976). In addition, *Heliconius* may also use green leaf volatiles during mate searching. Six-carbon alcohols and acetates are released in larger amounts after tissue damage by caterpillars, which males of the pupal mating species *H. charithonia* then use to find potential mates (Estrada & Gilbert, 2010). Once males find pupae they also use chemical cues to determine sex (Estrada *et al.*, 2010).

There are also a number of observations that indicate a role for chemical recognition during adult mating. *H. erato* males can distinguish between wings dissected from conspecific and heterospecific females that are virtually identical in wing pattern, but this effect disappears after wings have been washed in hexane (Estrada & Jiggins, 2008). *H. cydno* males show a preference for their own pattern over that of the closely related *H. melpomene*, but will court wing pattern models of *H. melpomene*. However, *H. cydno* males have virtually never been observed mating with *H. melpomene* females, suggesting strong barriers in addition to wing pattern (Naisbit, Jiggins, & Mallet, 2001). In addition, *H. melpomene* coexists with visually almost identical co-mimics, notably *H. erato*, which is likely to favour the evolution of pheromonal recognition signals to avoid confusion in courtship.

In some populations the very closely related species *H. timareta,* is sympatric and mimetic with *H. melpomene*, but nevertheless displays strong assortative mating (Giraldo *et al.*, 2008). A recent study of sympatric *H. melpomene* and *H. timareta* in Peru has provided some of the first evidence for a role of chemical signals in species recognition (Mérot *et al.*, 2015). Experiments with perfumed males using abdominal scent glands and wing extracts shows increased probability of inter-specific mating when males were perfumed with heterospecific extracts. In addition, chemical analysis of both abdominal glands and whole

wings provided evidence for differences between these closely related species in their chemical signatures (Mérot *et al.*, 2015)

Here we focus on the wing pheromones of *H. melpomene*. First, we investigate morphological structures potentially associated with pheromone production. In butterflies, a variety of species-specific structures including brushes, fans, and differentiated scales on wings, legs or abdomen are used to expose pheromones produced in associated glands (Wyatt, 2003; Nieberding *et al.*, 2008). In particular, male specific scent glands, termed androconia are common across the Lepidoptera. In male *Heliconius*, a patch of shiny grey scales is present on the overlapping region of the hind and forewing (Figure 1). The observed sexual dimorphism in this trait suggests that these are androconia, and may be associated with a male sex pheromone (Emsley, 1963). Furthermore, earlier authors have identified brush-like scales in this region that are the putative site for pheromone production (Müller, 1912; Barth, 1952). Here we investigate the structure of these scales using Scanning Electron Microscopy. Second, we complement recently published chemical analysis of whole *H. melpomene* wings by dissecting wing regions to identify those wing regions that are associated with the production of compounds and identify potential male sex pheromone compounds isolated from this region. Finally, using a simple behavioural assay we evaluate female response to male androconial pheromone extracts.

Methods

Individuals used for morphological and chemical analyses were from an outbred stock of *Heliconius melpomene plesseni* and *Heliconius melpomene malleti* (sold as *H. m. aglaope*) maintained at the University of Cambridge insectaries (Figure 1). These two races are from the region of a hybrid zone in the eastern Andes of Ecuador, and showed signs of inter-racial hybridization in the stocks, so are treated here as a single population and referred to as the Ecuador samples. These stocks were established from individuals obtained from a commercial breeder (*Stratford Butterfly Farm*: www.butterflyfarm.co.uk). Laboratory stocks were maintained on the larval food plants, *Passiflora menispermifolia* and *Passiflora biflora*. Adult butterflies were fed on ~10% sugar solution mixed with an amino acid supplement (Critical Care Formula®). In addition, five individual males of *H. m. rosina* were sampled from the wild around the town of Gamboa in Colón Province, Panama, and are referred to as the Panama samples.

Morphological analysis

The detailed morphology of androconial scales was determined using Field Emission Scanning Electron Microscope. Three males and two females of *H. melpomene* from Ecuador

137 were used for this analysis. The androconial grey scale region was dissected out from both
138 hind and forewings and attached to aluminium stubs with carbon tabs and subsequently
139 coated with 20nm of gold using a Quorum/Emitech sputter coater. The gold-coated
140 androconia were then viewed in an FEI XL30 FEGSEM operated at 5kV. Images were
141 recorded digitally using XL30 software at 500x magnification.
142
143 *Characterization of potential male sex pheromone*
144 Wing tissue from ten male (five newly emerged and five 10-day old mature individuals) and
145 five female (10-day old) individuals from the Ecuador stock was collected between November
146 2011 and March 2012 for chemical analysis. In addition, wing tissue from five male adult
147 individuals from Panama was collected in August 2012 for chemical analysis. Wings were
148 dissected into four parts: forewing androconia, hindwing androconia, forewing non-
149 androconia and hindwing non-androconia. The 'androconia' regions corresponded to the
150 grey-brown region shown in Figure 1c, with non-androconia corresponding to the remaining
151 portion of the wing. In females, a region corresponding in size and extent to the grey-brown
152 region seen in males was dissected. The dissected sections were then allowed to soak in either
153 hexane or dichloromethane for 3 hours in a glass vial. The solvent was then transferred to new
154 vial and stored at -20°C. Initial tests showed no major differences between hexane and
155 dichloromethane extracts. Therefore, the more polar dichloromethane was used in later
156 analysis.
157 Extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) using a
158 Hewlett-Packard model 5975 mass-selective detector connected to a Hewlett-Packard GC
159 model 7890A, and equipped with a Hewlett-Packard ALS 7683B autosampler. A HP-5MS
160 fused silica capillary column (Agilent, 30 m × 0.25 mm, 0.25 μm) was used. Injection was
161 performed in splitless mode (250°C injector temperature) with helium as the carrier gas
162 (constant flow of 1.2 ml/min). The temperature programme started at 50°C, was held for 5
163 min, and then rose to 320°C with a heating rate of 5°C/min. All components were identified
164 by comparison of mass spectra and gas chromatographic retention index with those of
165 authentic reference samples, and analysis of mass spectra. The double bond position of
166 unsaturated compounds were determined by derivatisation with dimethyl disulfide (Buser *et*
167 *al.*, 1983). The alcohols were synthesised from the corresponding esters by reduction
168 according to established procedures (Becker & Beckert, 1993, p. 570). The aldehydes were
169 synthesised by oxidation of the respective alcohols (More & Finney, 2002). Synthesis of the
170 methyl branched alcohols and aldehydes will be reported elsewhere (F. Mann et al., in
171 preparation). The samples were quantified by using gas chromatography with flame ionisation
172 detection with a Hewlett-Packard GC model 7890A equipped with a Hewlett-Packard ALS

7683B autosampler. A BPX-5 fused silica capillary column (SGE, 25 m × 0.22 mm, 0.25 µm) was used. Injection was performed in splitless mode (250°C injector temperature) with hydrogen as the carrier gas (constant flow of 1.65 ml/min). The temperature programme started at 50°C, held for 5 min, and then rose to 320°C with a heating rate of 5°C/min. Pentadecyl acetate (10.1 ng) or (*Z*)-4-tridecenyl acetate (1 ng) were used as internal standard. Only compounds eluting earlier than hexacosane were considered for analysis. Later compounds were identified as cuticular hydrocarbons, 2,5-dialkyltetrahydrofurans, cholesterol and artefacts like phthalates or adipates. The variability in the late eluting cuticular hydrocarbons was low and did not show characteristic differences.

Behavioural experiments

Behavioural assays to determine female response to the putative male sex pheromone were conducted in the *Heliconius* insectaries at the Smithsonian Tropical Research Institute's (STRI) in Gamboa, Panama between January 2013 and July 2013. Females were assayed from an outbred stock of *H. melpomene rosina* established from wild individuals collected in Gamboa (9°7.4′ N, 79°42.2′ W, elevation 60 m) and the nearby Soberanía National Park, República de Panamá. Stocks were maintained on the larval food plants, *Passiflora menispermifolia* and *Passiflora biflora*. Adult butterflies were fed on ~20% sugar solution with pollen supplements and *Psychotria sp*. and *Psiguiria sp*. serving as an additional pollen source. Males sacrificed for extracts for behavioural experiments were either from this same stock or were wild individuals collected in Gamboa or the Soberanía National Park. Male extracts were obtained by dissecting the androconial region from both hindwings, followed by soaking in 200 µl of hexane for 2-3 hours before transferring the solvent to a new glass vial. This vial was sealed and stored at -20°C until required.

Behavioural experiments were performed in cages approximately 2m x 2m x 2m. Ten virgin females (<5 days after eclosion) were presented with a paper model of *H. melpomene rosina* attached to a ~30cm length of wire, itself attached to a ~60cm stick on which a camera (Xdreme HD 1080P Action camera) was mounted to record female behaviour. Pheromone extracts from individual *H. melpomene* males or a control (solvent only) were applied to a strip of Whatman filter paper no. 4, which was attached to the paper butterfly model. The extract of a single male was used for each individual female, so in total ten virgin females and ten male extracts were used. Each trial proceeded as follows: After mounting the paper model on the wire, 200 µl of extract/control was applied to the filter paper. Trials were conducted blind: A third party applied the extract/control so that the experimenter did not know whether they were presenting the focal female with the extract or control. The paper model was then presented to the focal female, and the apparatus

manipulated to simulate the hovering flight observed in courting males. Each presentation lasted one minute, followed by a one-minute break when the model was removed from the vicinity of the focal female. This was repeated five times before a further five-minute pause in the trial. The whole procedure was repeated six times, alternating between the two treatments, so that each female experienced a total of 15 presentations with the male extract and 15 presentations with the control (solvent only).

Female behaviours were scored from videos recorded during the trials. To avoid observer bias, the names and order of the digital video files were randomized and only reassigned to treatment and individual once scoring was complete. We determined the behaviours to be recorded from observations of interactions between *H. melpomene* males and females (Table 1). These were 'Slow and moderate wing flapping', where females opened and closed their wings slowly at regular intervals displaying their wing colors; 'Flying/Flying facing towards model'where females tried to chase the paper models for short intervals or females hovered facing the model; 'Slow rhythmic flight' where females displayed slow flight with intermittent gliding; 'Wing display with abdomen normal' where females opened their wings displaying the dorsal wing surface with the abdomen between the wings; 'Quick and jerky wing flapping' where there was continuous wing fluttering by females that effectively prevented close contact by the model; 'Flying away from model' where females flew away from the paper model avoiding any interaction; 'Fast erratic flight' where such flight was notably very fast, abrupt and apparently directionless and finally 'Wings open with abdomen erect' where females opened their wings displaying the dorsal wing surface with the abdomen erect. Each of these behaviours was scored as having occurred or not occurred during each of the one-minute presentations. This led to a dataset for individual females where the proportion of one-minute presentations in which each behaviour was observed was calculated (for male extract and control trials separately). To reduce the number of dependent variables we then performed a principal component analysis on these data. The principal component scores for each individual were extracted and were used to test for differences in response between presentations with the male extract and control (solvent only) in paired tests. Statistical analyses were performed with *R* (version 3.1.2).

Results

Morphological analysis

In order to investigate structural differences in scale morphology potentially associated with pheromone production, we observed androconial scales of *H. melpomene rosina* males and females under the scanning electron microscope (Figure 2). We identified a marked sexual dimorphism in scale structure. In the central region of the male hindwing androconia along

245 vein Sc+R1 we identified scales with brush-like structures (Figure 2d) at their distal end
246 (Figure 2a). These scales were completely absent in females and in the forewing androconia
247 of the males (Figure 2). These scales were also not detected in any other wing region
248 examined. The brush-like scales were found in alternating rows with scales with a normal
249 structure. Moving away from the Sc+R1 wing vein, the density and width of these scales
250 decreased, with isolated brush-like scales found completely surrounded by normal scales. In
251 addition, it was found that the base of these brush-like scales was more swollen and glandular
252 as compared to other scales, perhaps indicating a location for the storage or production of the
253 pheromone (Figure 3).
254
255 *Characterization of potential male sex pheromone*
256 Using gas chromatography linked to mass spectrometry (GC/MS) and synthesis, six potential
257 male sex pheromone components were found in the male wing extracts from Ecuador samples
258 (Figure 4). These compounds were, (*Z*)-9-octadecenal, octadecanal, henicosane, (*Z*)-11-
259 icosenal, icosanal and (*Z*)-13-docosenal. Out of these six compounds, only henicosane was
260 found in all regions of the wing, but (*Z*)-9-octadecenal, octadecanal, (*Z*)-11-icosenal, icosanal
261 and (*Z*)-13-docosenal were restricted to the androconial scale region of the wing. The
262 hindwing androconial region tended to contain higher titres of these five compounds as
263 compared to forewing androconial wing region (Figure 5). Octadecanal was present in higher
264 amounts in hindwing androconia, while icosanal were only found in trace quantities. (Z)-9-
265 Octadecenal was found in small amounts only in some of the Ecuador samples. A related
266 compound, (*Z*)-11-Icosanol, was found in trace amounts in two Ecuador mature males. Except
267 for henicosane, all five compounds were observed to be age-specific and sex-specific.
268 Henicosane was present in the hindwing androconia of both 10-day old and newly emerged
269 males and in females of all ages. In contrast, (*Z*)-9-octadecenal, octadecanal, (*Z*)-11-icosenal,
270 icosanal and (*Z*)-13-docosenal were only present in extracts from the hindwing androconia of
271 10-day old males. The chemical analysis of the extracts of the Panama samples showed a
272 similar composition, although they contained more compounds in slightly higher
273 concentrations. Major components of both the Panama and Ecuador individuals were
274 octadecanal, (*Z*)-11-icosenal, icosanal, and (*Z*)-13-docosenal. (*Z*)-11-Icosenol was found in
275 larger amounts in the Panama samples. They additionally contained high amounts of
276 octadecanol. Small amounts of nonadecanal, methyl-branched octadecanals and their
277 respective alcohols occurred as well. Some of them were identified to be 15-, 16-, and 17-
278 methyloctadecanals and the respective alcohols. These compounds were found only in the
279 Panama samples with the exception of 17-methyloctadecanal, which was present in two of the
280 samples from Ecuador. *n*-Alkanes were not present in these extracts.

281

282 *Behavioural experiments*

283 We tested ten individual virgin females in our behavioural trials, each of which was presented
284 sequentially with either male wing extract or a control (solvent only) applied to a paper
285 model. Behavioural scores were analysed using a principal components analysis, with two
286 principal components retained that together accounted for 80.38% of the variance in the data
287 (Table 1, see also supplementary file 1 for the raw data). The second axis accounted for
288 18.3% of the variance. There was no significant difference between male extract and control
289 (solvent only) trials for PC1 (Figure 6; Wilcoxon signed rank test: $V = 33$, $p > 0.05$);
290 however, there was a significant difference between male extract and control (solvent only)
291 trials for PC2 (Figure 6; Wilcoxon signed rank test: $V = 49$, $p < 0.05$).

292

293 **Discussion**

294 Many previous studies of *Heliconius* butterflies have described a role for visual cues in mate
295 finding, courtship behaviour, reproductive isolation and speciation (Crane, 1955; Brown, 1981;
296 Jiggins *et al.*, 2001; Kronforst *et al.*, 2006; Giraldo *et al.*, 2008; Estrada & Jiggins, 2008; Merrill
297 *et al.*, 2011a). Here, we have identified compounds associated with sexually mature male wings
298 and described morphological structures putatively involved in pheromone release. Furthermore,
299 we have shown that wing extracts influence female behaviour.

300 We identified six potential MSP compounds from male wing extracts, namely, (*Z*)-9-
301 octadecenal, octadecanal, henicosane, (*Z*)-11-icosenal, icosanal and (*Z*)-13-docosenal. Five of
302 these are restricted to the androconial region of hindwings of 10-day old males, suggesting a
303 likely role in courtship. Our results are broadly comparable with another recent analysis of wing
304 compounds in *Heliconius* (Mérot *et al.*, 2015), although this recent study did not compare
305 different wing regions, or similarly aged males and females. As the study also did not use
306 synthesis to identify compounds, our work is highly complementary and extends their results
307 to confirm localization of compounds to older males. Male *Heliconius* do not become sexually
308 active until several days after eclosion, so the absence of these compounds from females and
309 younger males is strongly suggestive of a role in mating behaviours. That these five MSP
310 compounds are largely restricted to the hindwing androconia of mature males (Figure 5a)
311 suggests that pheromone storage or production is restricted to the hindwing. Trace amounts on
312 the forewing androconia may be due to contact in the overlapping portion of the fore- and
313 hindwings, and both wings may play a role in dispersal of the compounds during courtship. The
314 wild samples from Panama had both a greater diversity of compounds and higher concentrations
315 on their wings. Further work will be needed to determine whether this is characteristic of natural

populations, or reflects a difference between geographic populations of *H. melpomene*, or is perhaps simply a result of natural inter-individual variation.

Scanning electron microscope images of the androconia support storage and/or production of MSPs in the hindwing. The androconial region of male hindwings are equipped with special brush-like scales (Figure 2a), which might facilitate the release of the pheromone during courtship and are completely absent in females and any other region of male wings. These were located primarily around and along the hindwing vein Sc+R1, similar to the depiction in Figure 73 of Emsley's previous morphological analysis (Emsley, 1963). Similar scales have been described from light microscopy in other *Heliconius* species, but not previously in *H. melpomene* (Müller, 1912; Barth, 1952). The base of these special brush-like scales was more swollen and glandular as compared to other scales (Figure 3), perhaps indicating a role in storage or production of pheromones by these scales. Even though *Heliconius* do not have the dramatic sexual dimorphism in hair pencils or brush-like androconia seen in other butterflies, they nonetheless do possess male-specific structures likely associated with pheromone production.

We were able to detect a significant, though subtle, difference in the behavioural response of females towards models treated with male wing extracts as compared to an unscented control. This indicates that females both detect compounds found on the male wings, and alter their behaviour in response to those compounds. This supports our hypothesis that these compounds act as a pheromone involved in courtship behaviour. Nonetheless, it remains unclear exactly what the information is that is conveyed by these signals. The signal may influence female courtship, although it is likely that these compounds convey complex information about male species identity, quality, age etc. that are interpreted by females in combination with visual and tactile cues. Recent experiments carried out in Peru also demonstrate the role of chemical signals in species recognition between *H. melpomene* and *H. timareta*, although these do not distinguish between the role of wing and abdominal pheromone signals (Mérot *et al.*, 2015). Further experiments are necessary to disentangle the details of inter-specific signaling during courtship in *Heliconius*. We are also currently unable to determine which of the compounds identified here are biologically active. Experiments with synthetic blends of putative MSP compounds were largely inconclusive, so are not presented here.

The use of multiple signals is common in animal communication (Candolin, 2003). Moths being nocturnal mainly depend upon chemical cues to attract their mates (Ando, Inomata, & Yamamoto, 2004). On the other hand, butterflies primarily use visual cues to locate mates (Kemp & Rutowski, 2011). It has been shown in *B. anynana* that in addition to visual cues, chemical cues also play a role and are equally important in sexual selection by female

choice (Costanzo & Monteiro, 2007). Divergent color patterns and extensive mimicry by different species of *Heliconius* has resulted in the use of multiple signals to maintain species specificity. Two sympatric cryptic species that share a wing pattern, *H. melpomene malleti* and *H. timareta florencia*, nonetheless show strong assortative mating. This suggests that enhanced divergence in pheromonal signals acts as an important cue in reproductive isolation in these species (Giraldo *et al.*, 2008). Exploring the pheromonal signals and morphological structures involved in the process in *H. melpomene* is our first step towards understanding multimodal signaling and its role in reproductive isolation.

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References

Ando T, Inomata S ichi, Yamamoto M. 2004. Lepidopteran Sex Pheromones. In: Schulz S, ed. Topics in Current Chemistry. The Chemistry of Pheromones and Other Semiochemicals I. Springer Berlin Heidelberg, 51–96.

Bacquet PMB, Brattström O, Wang HL, Allen CE, Löfstedt C, Brakefield PM, Nieberding CM. 2015. Selection on male sex pheromone composition contributes to butterfly reproductive isolation. *Proceedings. Biological Sciences / The Royal Society* **282**: 20142734.

Barth R. 1952. Os órgãos odoriferos masculinos de alguns Heliconiinae do Brasil. *Memórias do Instituto Oswaldo Cruz* **50**: 355–367.

Bates HW. 1862. Contributions to an insect fauna of the Amazon valley. Lepidoptera: Heliconidae. *Transactions of the Linnean Society of London* **23**: 495–566.

Becker HG, Beckert R. 1993. *Organikum-Organisch-Chemisches Praktikum*. Wiley-VCH Weinheim.

Brower LP, Jones MA. 1965. Precourtship interaction of wing and abdominal sex glands in male Danaus butterflies. *Proceedings of the Royal Entomological Society of London. Series A, General Entomology* **40**: 147–151.

Brown KS. 1981. The Biology of Heliconius and Related Genera. *Annual Review of Entomology* **26**: 427–456.

Buser HR, Arn H, Guerin P, Rauscher S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Analytical Chemistry* **55**: 818–822.

390 **Candolin U. 2003**. The use of multiple cues in mate choice. *Biological Reviews* **78**: 575–
391 595.

392 **Costanzo K, Monteiro A. 2007**. The use of chemical and visual cues in female choice in
393 the butterfly *Bicyclus anynana*. *Proceedings of the Royal Society of London B: Biological*
394 *Sciences* **274**: 845–851.

395 **Crane J. 1955**. Imaginal behaviour of a Trinidad butterfly, *Heliconius erato hydara*
396 Hewitson, with special reference to the social use of color. *Zoologica, New York* **40**: 167–
397 196.

398 **Emsley MG. 1963**. A morphological study of imagine Heliconiinae (Lep., Nymphalidae)
399 with a consideration of the evolutionary relationships within the group. *Zoologica, New*
400 *York* **48**: 85–130.

401 **Estrada C, Yildizhan S, Schulz S, Gilbert LE. 2010**. Sex-specific chemical cues from
402 immatures facilitate the evolution of mate guarding in *Heliconius* butterflies. *Proceedings*
403 *of The Royal Society. Biological Sciences* **277**: 407–413.

404 **Estrada C, Gilbert LE. 2010**. Host plants and immatures as mate-searching cues in
405 Heliconius butterflies. *Animal Behaviour* **80**: 231–239.

406 **Estrada C, Jiggins CD. 2008**. Interspecific sexual attraction because of convergence in
407 warning colouration: is there a conflict between natural and sexual selection in mimetic
408 species? *Journal of Evolutionary Biology* **21**: 749–60.

409 **Finkbeiner SD, Briscoe AD, Reed RD. 2014**. Warning signals are seductive: Relative
410 contributions of color and pattern to predator avoidance and mate attraction in
411 *Heliconius* butterflies. *Evolution* **68**: 3410–3420.

412 **Gilbert LE. 1976**. Postmating female odor in Heliconius butterflies: a male-contributed
413 antiaphrodisiac? *Science* **193**: 419–420.

414 **Giraldo N, Salazar C, Jiggins CD, Bermingham E, Linares M. 2008**. Two sisters in the
415 same dress: *Heliconius* cryptic species. *BMC Evolutionary Biology* **8**: 324.

416 **Grillet M, Dartevelle L, Ferveur JF. 2006**. A Drosophila male pheromone affects female
417 sexual receptivity. *Proceedings of the Royal Society of London B: Biological Sciences* **273**:
418 315–323.

419 **Grula JW, McChesney JD, Taylor OR. 1980**. Aphrodisiac pheromones of the sulfur
420 butterfliesColias eurytheme andC. Philodice (Lepidoptera, Pieridae). *Journal of Chemical*
421 *Ecology* **6**: 241–256.

422 **Jiggins CD, Naisbit RE, Coe RL, Mallet J. 2001**. Reproductive isolation caused by colour
423 pattern mimicry. *Nature* **411**: 302–305.

424 **Jiggins CD. 2008**. Ecological Speciation in Mimetic Butterflies. *BioScience* **58**: 541–548.

425 **Jiggins CD, Estrada C, Rodrigues A. 2004**. Mimicry and the evolution of premating
426 isolation in *Heliconius melpomene* Linnaeus. *Journal of Evolutionary Biology* **17**: 680–
427 691.

428 **Kemp DJ, Rutowski RL. 2011**. The Role of Coloration in Mate Choice and Sexual
429 Interactions in Butterflies. Advances in the Study of Behavior. Elsevier, 55–92.

Kronforst MR, Young LG, Kapan DD, McNeely C, O'Neill RJ, Gilbert LE. 2006. Linkage of butterfly mate preference and wing color preference cue at the genomic location of wingless. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 6575–6580.

Lassance JM, Groot AT, Liénard MA, Antony B, Borgwardt C, Andersson F, Hedenström E, Heckel DG, Löfstedt C. 2010. Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones. *Nature* **466**: 486–489.

Löfstedt C. 1993. Moth Pheromone Genetics and Evolution. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **340**: 167–177.

Meinwald J, Meinwald YC, Mazzocchi PH. 1969. Sex Pheromone of the Queen Butterfly: Chemistry. *Science* **164**: 1174–1175.

Melo MC, Salazar C, Jiggins CD, Linares M. 2009. Assortative mating preferences among hybrids offers a route to hybrid speciation. *Evolution* **63**: 1660–1665.

Mérot C, Frérot B, Leppik E, Joron M. 2015. Beyond magic traits: Multimodal mating cues in *Heliconius* butterflies. *Evolution*: n/a–n/a.

Merrill RM, Gompert Z, Dembeck LM, Kronforst MR, McMillan WO, Jiggins CD. 2011a. Mate preference across the speciation continuum in a clade of mimetic butterflies. *Evolution* **65**: 1489–1500.

Merrill RM, Van Schooten B, Scott JA, Jiggins CD. 2011b. Pervasive genetic associations between traits causing reproductive isolation in Heliconius butterflies. *Proceedings of The Royal Society. Biological Sciences* **278**: 511–518.

Merrill RM, Dasmahapatra KK, Davey JW, Dell'Aglio DD, Hanly JJ, Huber B, Jiggins CD, Joron M, Kozak KM, Llaurens V, *et al.* 2015. The diversification of *Heliconius* butterflies: what have we learned in 150 years? *Journal of Evolutionary Biology* **28**: 1417–1438.

Merrill RM, Chia A, Nadeau NJ. 2014. Divergent warning patterns contribute to assortative mating between incipient *Heliconius* species. *Ecology and Evolution* **4**: 911–917.

More JD, Finney NS. 2002. A Simple and Advantageous Protocol for the Oxidation of Alcohols with o-Iodoxybenzoic Acid (IBX). *Organic Letters* **4**: 3001–3003.

Müller F. 1912. X. The Scent-scales of the Male 'Maracujá butterflies'. In: Longstaff GB, ed. Butterfly Hunting in Many Lands. New York: Longmans, Green & Co., 655–659.

Naisbit RE, Jiggins CD, Mallet J. 2001. Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. *Proceedings of the Royal Society of London Series B-Biological Sciences* **268**: 1849–1854.

Nieberding CM, de Vos H, Schneider MV, Lassance JM, Estramil N, Andersson J, Bång J, Hedenström E, Löfstedt C, Brakefield PM. 2008. The Male Sex Pheromone of the Butterfly *Bicyclus anynana*: Towards an Evolutionary Analysis. *PLoS ONE* **3**: e2751.

Nieberding CM, Fischer K, Saastamoinen M, Allen CE, Wallin EA, Hedenström E, Brakefield PM. 2012. Cracking the olfactory code of a butterfly: the scent of ageing. *Ecology Letters* **15**: 415–424.

Nishida R, Schulz S, Kim CS, Fukami H, Kuwahara Y, Honda K, Hayashi N. 1996. Male sex pheromone of a giant danaine butterfly, *Idea leuconoe*. *Journal of Chemical Ecology* **22**: 949–972.

Phelan PL, Baker TC. 1987. Evolution of male pheromones in moths: reproductive isolation through sexual selection? *Science (New York, N.Y.)* **235**: 205–207.

Pliske TE, Eisner T. 1969. Sex Pheromone of the Queen Butterfly: Biology. *Science* **164**: 1170–1172.

Schulz S, Estrada C, Yildizhan S, Boppré M, Gilbert LE. 2008. An Antiaphrodisiac in Heliconius melpomene Butterflies. *Journal of Chemical Ecology* **34**: 82–93.

Smadja C, Butlin RK. 2008. On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity* **102**: 77–97.

Symonds MRE, Johnson TL, Elgar MA. 2012. Pheromone production, male abundance, body size, and the evolution of elaborate antennae in moths. *Ecology and Evolution* **2**: 227–246.

Wyatt TD. 2003. *Pheromones and Animal Behaviour: Communication by Smell and Taste*. Cambridge University Press.

Wyatt TD. 2014. *Pheromones and Animal Behavior: Chemical Signals And Signatures*. Cambridge: Cambridge University Press.

Yildizhan S, van Loon J, Sramkova A, Ayasse M, Arsene C, ten Broeke C, Schulz S. 2009. Aphrodisiac Pheromones from the Wings of the Small Cabbage White and Large Cabbage White Butterflies, *Pieris rapae* and *Pieris brassicae*. *ChemBioChem* **10**: 1666–1677.

Figure 1. A) *H. melpomene malleti* male (left) and *H. melpomene plesseni* female (right). B) Dissected wings from specimens of *H. melpomene plesseni* showing sexual dimorphism in the androconial region, with male (left) and female (right). C) Expanded view of the androconial region with arrow highlighting the vein Sc+R1. The pale grey-brown region in the male wing was dissected for chemical analysis.

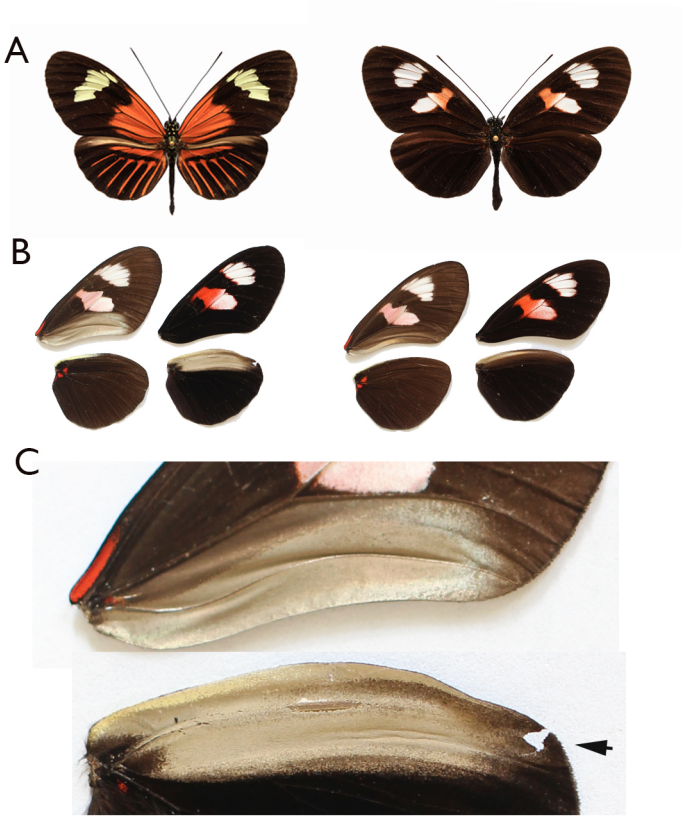

Figure 2: SEM image of putative androconial scales of *H. melpomene.* Scales in the region of the hindwing vein Sc+R1 and forewing vein 1A are shown. (A) male hindwing (B) male forewing and (C) female forewing at 500x magnification. (D) Magnified view of brush-like structures of the special scales. Scale bars indicate 50 µm (A-C) and 2 µm (D).

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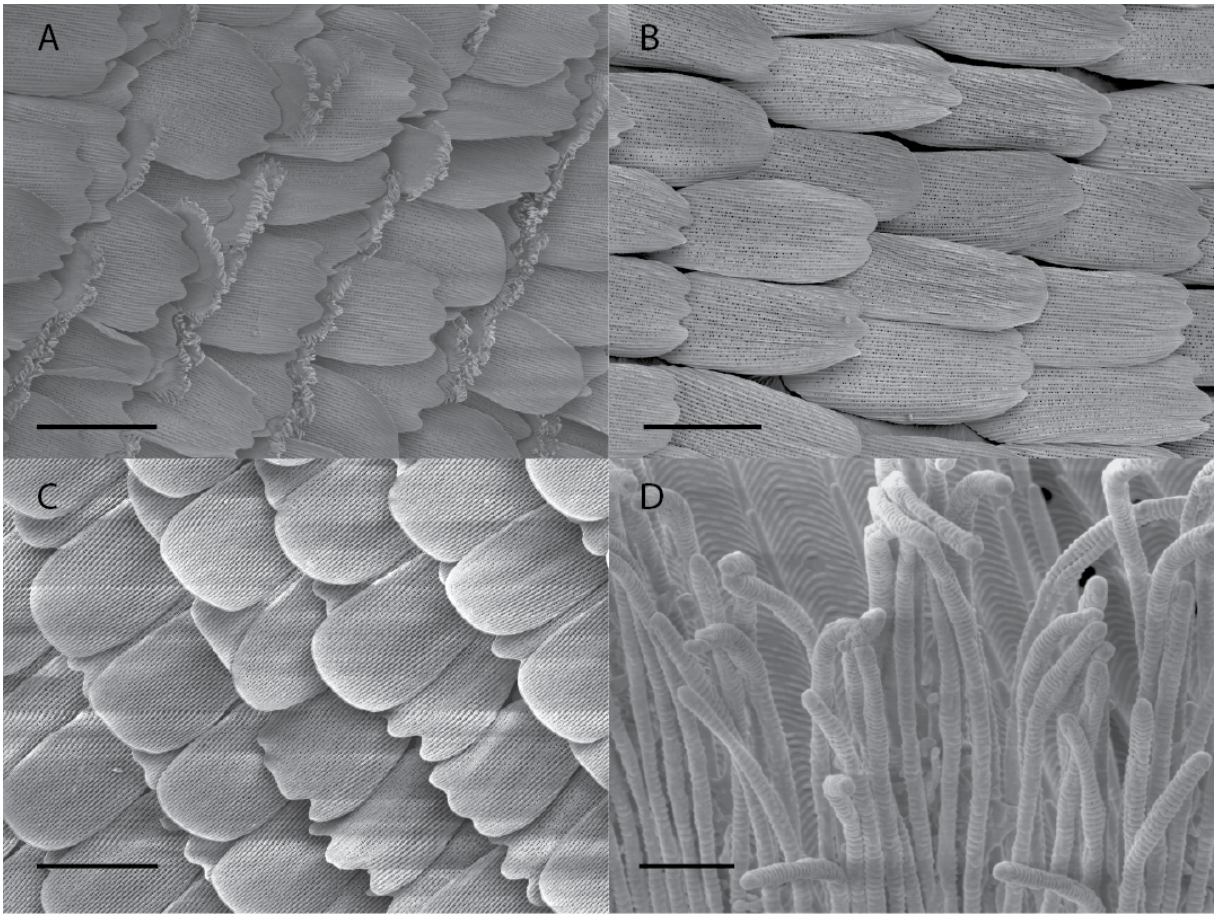


Figure 3: SEM image of the base of a scale of hindwing androconia of *H. melpomene* (A) scale from androconial region of male with brush-like structures. (B) scale from androconial region of female. Scale bars indicate 10 µm.

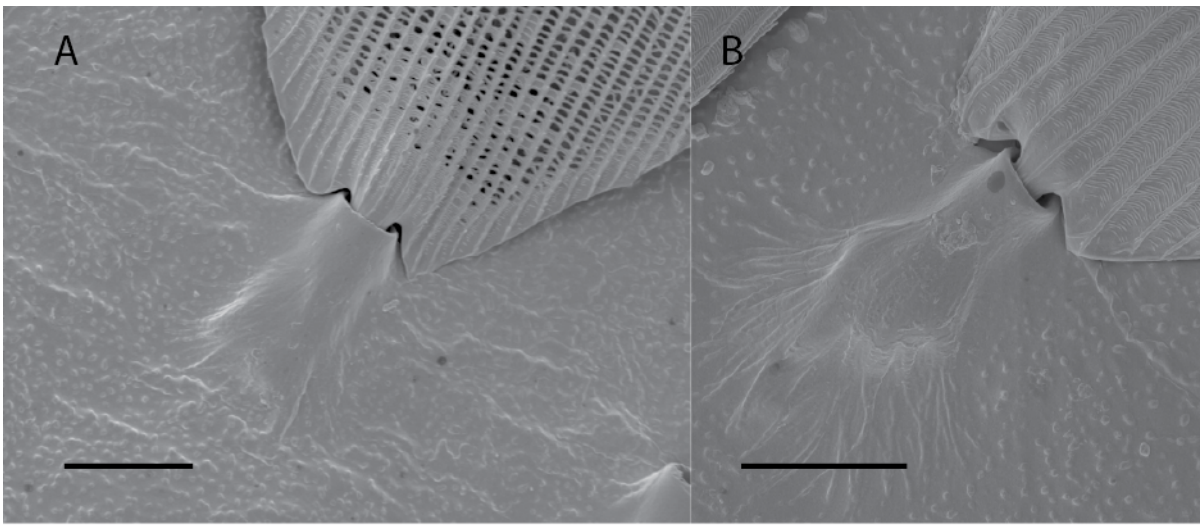
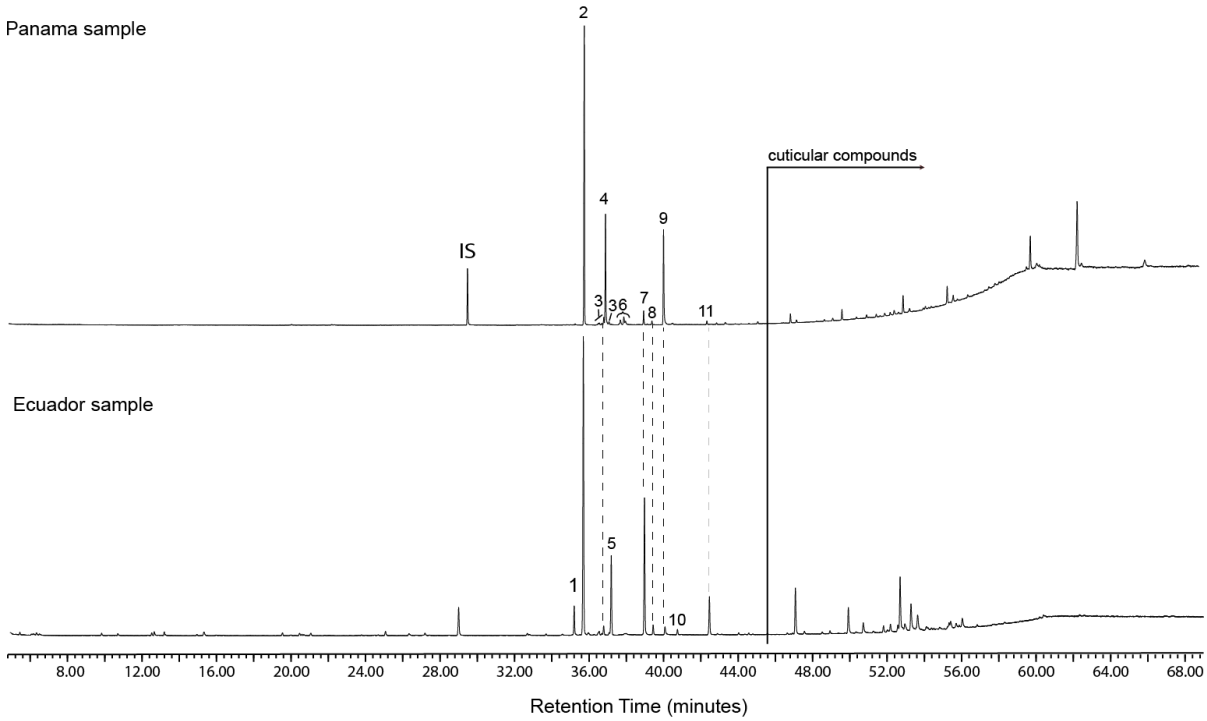
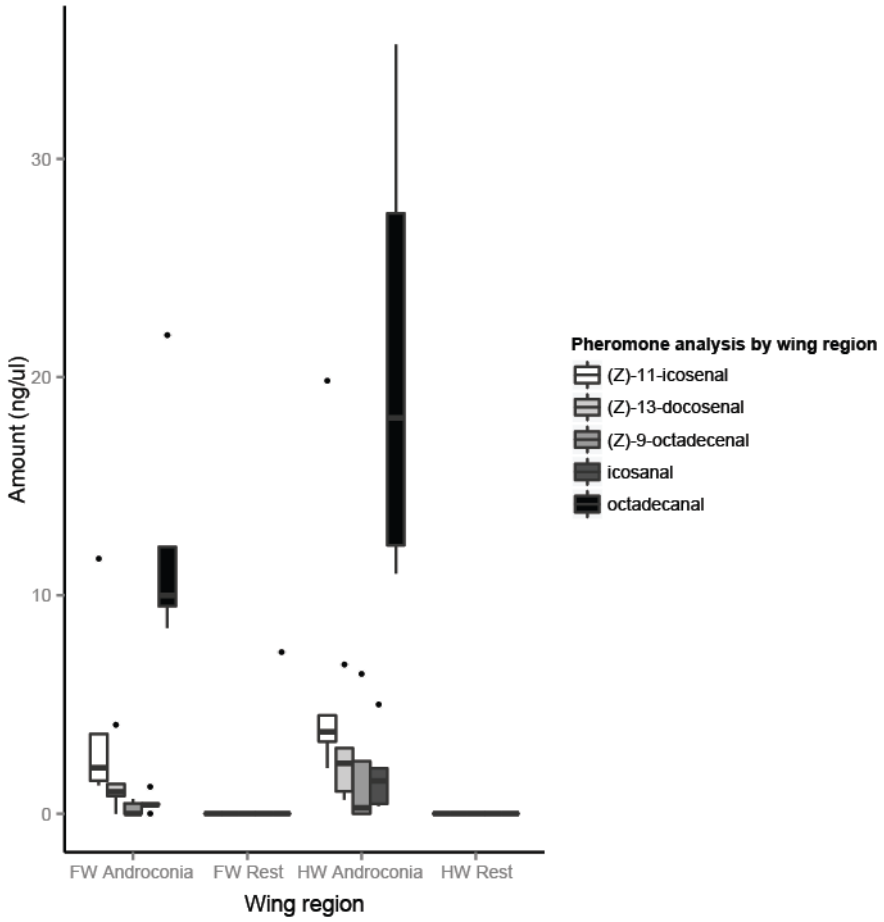


Figure 4: Total ion chromatograms of extracts from the androconial region of an Ecuadorian *H. melpomene* and a Panamanian *H. melpomene* male hindwing. 1: (*Z*)-9-octadecenal; 2: octadecanal; 3: 15-, 16-, 17-methyloctadecanals, and additional methyloctadecanal; 4: 1-octadecanol; 5: henicosane; 6: 15-, 16-, and 17-methyloctadecan-1-ols, additional methyloctadecanol, and nonadecanal; 7: (*Z*)-11-icosenal; 8: icosanal; 9: (*Z*)-11-icosenol; 10: tricosane; 11: (*Z*)-13-docosenal. All peaks eluting later than 44 min are cuticular compounds consisting of larger *n*-alkanes, 2,5-dialkyltetrahydrofurans, cholesterol or are contaminations.

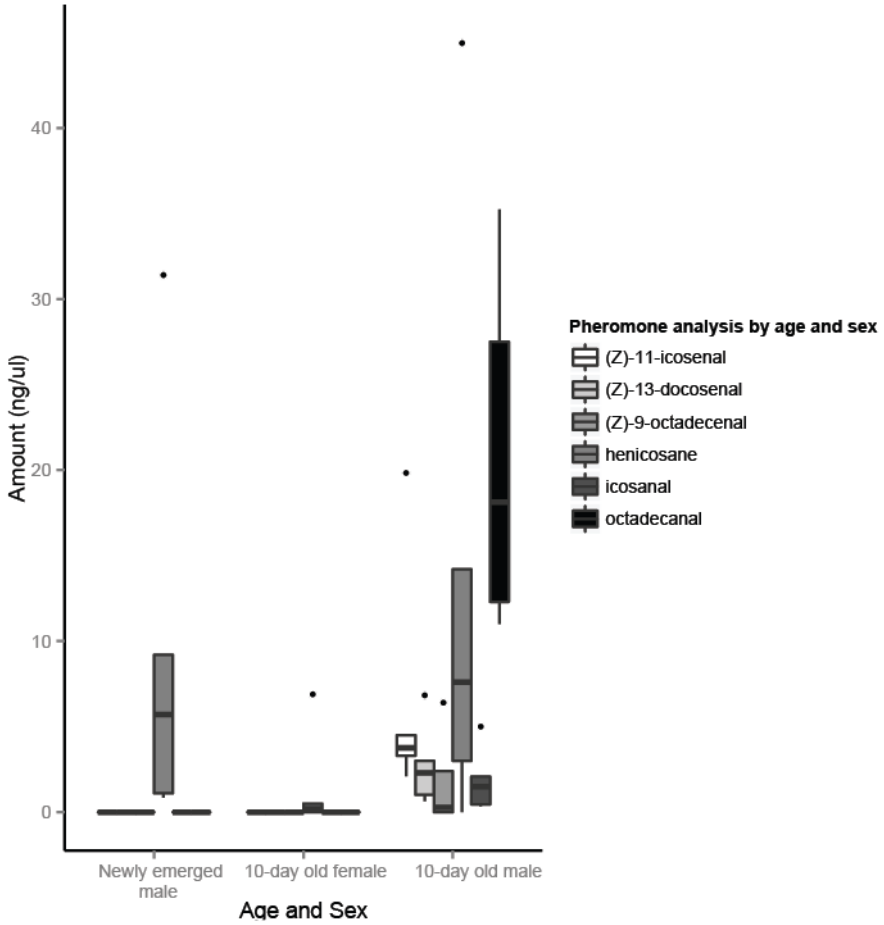


525 Figure 5: GC/MS analyses of *H. melpomene* wing extracts showing presence of putative male
526 sex pheromone compounds (a) Presence of compounds in the hindwing androconial region of
527 five mature females, five young and five mature males, showing that compounds are largely
528 restricted to mature males. (b) Presence of compounds in five mature females, five young and
529 five mature males, showing that compounds are largely restricted to mature males. (c)
530 Presence of compounds in hindwing androconial region of five wild males from Panama.

A

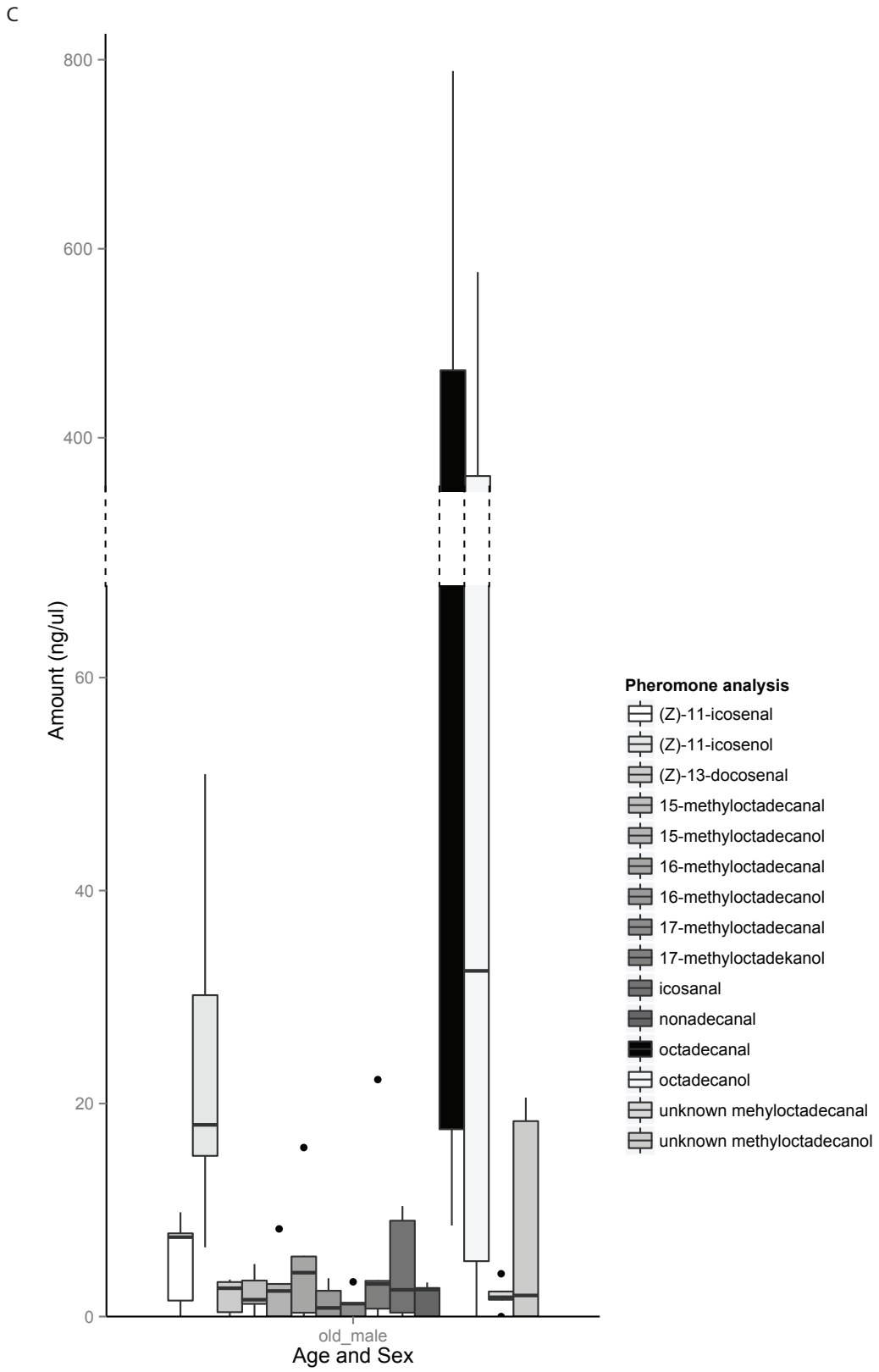

Amount (ng/ul)
30
20
10
0
FW Androconia
FW Rest
HW Androconia
HW Rest
Wing region
Pheromone analysis by wing region
(Z)-11-icosenal
(Z)-13-docosenal
(Z)-9-octadecenal
icosanal
octadecanal

B


Amount (ng/ul)
40
30
20
10
0
Newly emerged male
10-day old female
10-day old male
Age and Sex
Pheromone analysis by age and sex
(Z)-11-icosenal
(Z)-13-docosenal
(Z)-9-octadecenal
henicosane
icosanal
octadecanal

531

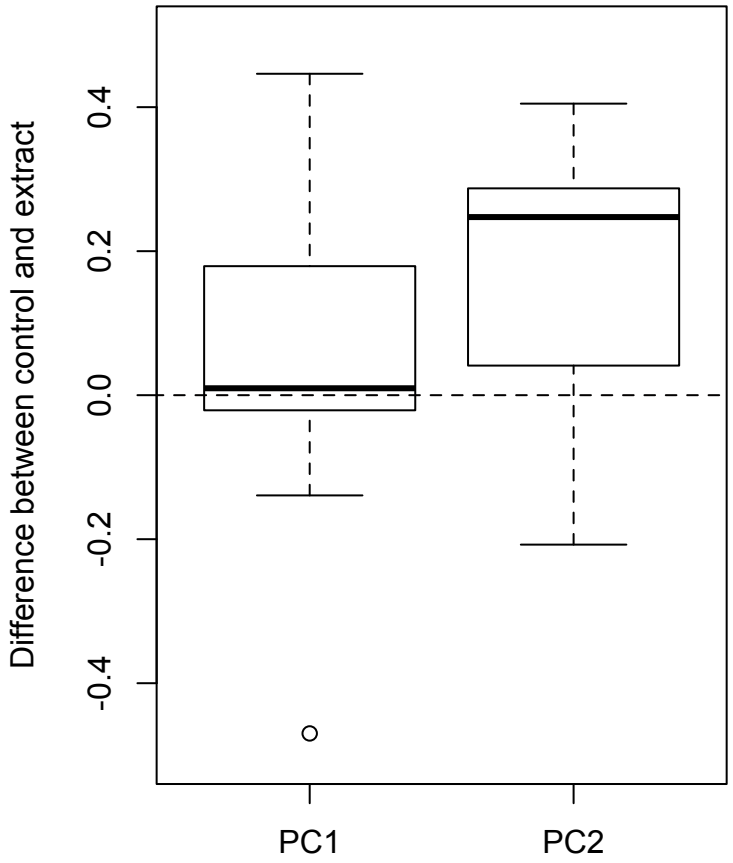
532


C
800
600
400
60
40
20
0
Amount (ng/ul)
old_male
Age and Sex
Pheromone analysis
(Z)-11-icosenal
(Z)-11-icosenol
(Z)-13-docosenal
15-methyloctadecanal
15-methyloctadecanol
16-methyloctadecanal
16-methyloctadecanol
17-methyloctadecanal
17-methyloctadekanol
icosanal
nonadecanal
octadecanal
octadecanol
unknown mehyloctadecanal
unknown methyloctadecanol

533

534

535 Figure 6: Differences in behavioural response for *H. melpomene* females presented with
536 models scented with male androconial extracts and unscented controls. Loading scores for
537 principal components are given in Table 1.



538
539

540 Table 1: Behavioural events scored for females presented with model butterflies and loading
541 scores for *H. melpomene* female response to differential treatments. The mean proportion of
542 trials in which a given behavior was observed is shown for both Control and Extract models.
543 The full data set is given as a supplementary file.
544

Observed behaviour	PC1	PC2	Ctrl	Extract
Slow and moderate wing flapping	0.15	-0.08	0.92	0.87
Flying/Flying facing towards model	-0.62	-0.12	0.45	0.41
Slow rhythmic flight	-0.41	-0.26	0.23	0.31
Wing display with abdomen normal	0.25	-0.63	0.09	0.15
Quick and jerky wing flapping	0.60	0.11	0.60	0.53
Flying away from model	-0.05	0.11	0.98	0.96
Fast erratic flight	-0.09	0.70	0.35	0.19
Wings open with abdomen erect	-0.03	0.09	0.04	0.04

545