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CARLOS EDUARDO BESERRA NOBRE DE ALMEIDA

O PAPEL DE SEMIOQUÍMICOS MASCULINOS NA DIFERENCIAÇÃO INTER- E INTRAESPECÍFICA DE BORBOLETAS (LEPIDOPTERA: PAPILIONOIDEA) DA FLORESTA ATLÂNTICA BRASILEIRA

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Tese apresentada ao Programa de Pós-Graduação em Biologia Animal da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutor em Biologia Animal

Área de Concentração: Ecologia

Orientador: Prof. Dr. Artur Campos Dália Maia

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RESUMO

Estímulos visuais e químicos desempenham papéis complementares no comportamento sexual de borboletas. Enquanto pistas visuais estão envolvidas em diversas etapas das interações intraespecíficas desses animais, a utilização de sinais químicos normalmente se restringe à comunicação em curtas distâncias. A produção e emissão de compostos químicos destinados à atração de indivíduos do sexo oposto normalmente ocorre em estruturas masculinas especializadas denominadas androcônias, as quais se apresentam como aglomerados de escamas morfologicamente diferenciadas. Borboletas do clado *Colias* (Pieridae: Coliadinae) e das tribos Heliconiini e Ithomiini (Nymphalidae), apresentam androcônias nas áreas de sobreposição das asas anteriores com as posteriores, mas seu arranjo e estrutura divergem. Compostos produzidos nas androcônias são potencialmente espécie-específicos e sua composição química pode estar relacionada à relação filogenética das espécies. Este estudo teve como objetivo investigar a composição química de secreções androconiais de borboletas do clado *Colias* (Pieridae: Coliadinae) e das tribos Ithomiini e Heliconiini (Nymphalidae: Danainae e Heliconiinae), relacionando-a a aspectos de sua morfologia alar, taxonomia e diversidade em diferentes sub-regiões da floresta atlântica brasileira. Compostos androconiais foram isolados através de cromatografia gasosa associada à espectrometria de massas. Matrizes de similaridade foram elaboradas a partir dos totais relativos dos compostos, seguidas de plotagem em gráficos de NMDS. Análises de ANOSIM foram usadas para identificar diferenças de perfis químicos entre táxons e entre categorias geográficas. Análises de regressão e teste de Mantel foram utilizados para testar, respectivamente, influências geográficas e genéticas sobre a composição androconial. As áreas androconiais das borboletas do clado *Colias* diferenciam-se claramente das regiões não androconiais das asas e exibem características morfológicas que podem atuar na prevenção da volatilização como na liberação de semioquímicos, tais como alta densidade e comprimento de escamas e padrão de perfuração destas. Em geral, a composição química androconial das borboletas estudadas é espécie-específica. Diferenças significativas também foram observadas entre os perfis químicos de duas subespécies de *Mechanitis lysimnia*, o que aponta que ambas podem estar em processo de isolamento. Para os compostos androconiais dos ninfalídeos estudados, observaram-se diferenças relacionadas a centros de endemismo, refúgios pleistocênicos e populações as quais não foram tão expressivas como as diferenças obtidas entre espécies. Correlações negativas entre distância geográfica e similaridade química foram discretas, mas presentes em todas as espécies de Ithomiini e Heliconiini. Uma correlação negativa entre distância genética e

similaridade química foi obtida para os Coliadinae. A categoria de espécie é a principal preditora de divergência química androconial e a natureza altamente espécie- e sexo-específica dos compostos indicam que estes agem como feromônios sexuais, estando envolvidos no processo de isolamento reprodutivo. Centros de endemismo da floresta Atlântica e áreas que correspondem aos refúgios pleistocênicos não predizem divergência química androconial melhor do que populações, o que sugere que os tempos de isolamento daquele período não foram suficientes para estabelecer um isolamento reprodutivo definitivo.

Palavras-chave: Características ligadas ao sexo. Comunicação química. Diversidade química. Feromônios masculinos. *Heliconius*. Papilionoidea.

ABSTRACT

Visual and chemical stimuli play complementary roles in the sexual behaviour of butterflies. While visual cues are involved in many stages of intraspecific interactions, chemical signals are usually restricted to short distance communication. Production and emission of chemical compounds destined to recognition and attraction normally take place in androconia. These are unique structures which can be identified as dense patches of specialized scales. Butterflies of the *Colias*-clade (Pieridae: Coliadinae) and of the tribes Heliconiini and Ithomiini (Nymphalidae) have androconial patches on the overlapping areas of their wings but scale arrangement and structure differ according to species. Chemicals produced in the androconia are potentially species-specific and there are indications that blend composition may be phylogenetically related. The objective of this study was to investigate the chemical composition of androconial secretions of *Colias*-clade (Pieridae: Coliadinae), Ithomiini and Heliconiini (Nymphalidae: Danainae e Heliconiinae) butterflies in different subregions of the Brazilian Atlantic forest and to relate the chemical similarities to aspects of alar morphology, taxonomy, and diversity. Androconial compounds were isolated through gas chromatography linked to mass spectrometry. Chromatogram peak areas were used to determine relative percentages, from which similarity matrices were performed followed by NMDS plotting. Differences among chemical profiles within *taxa* and among area-related categories were tested with ANOSIM analyses. Geographical and genetic influences over androconial composition was tested through regression analyses and Mantel tests, respectively. Androconial patches from all *Colias*-clade butterflies were differentiated from the non-androconial male wing surface and exhibited morphological features that may act in both preventing the volatilization of secretions and facilitating the release of semiochemicals, such as high density, length, and perforations of scales. The overall chemical composition of butterflies was highly species-specific. Large differences were also obtained between two subspecies of *Mechanitis lysimnia*, which point towards distinct species. Significant area-related differences occurred for populations, endemism centers and refugia for the androconial blends of nymphalids but not as high as species-related divergences. Negative correlations between geographical distance and chemical similarity were discrete but present in all species of Ithomiini and Heliconiini. A negative correlation was obtained between genetic distance and the androconial chemical similarity of the *Colias*-clade butterflies. Species are the main predictors of androconial chemical divergence and their highly species- and sex-specific nature indicates that they act sex pheromones and are involved in the reproductive isolation process. Atlantic forest endemism

centers and areas corresponding to Pleistocenic refugia do not predict androconial divergence better than populations alone, which suggest that isolation times from that period were not enough to establish a strong reproductive isolation.

Keywords: Butterfly communication. Chemical diversity. *Heliconius.* Male pheromone. Papilionoidea. Sex-related traits.

SUMÁRIO

1 INTRODUÇÃO

Borboletas e mariposas fazem parte da classe animal que provavelmente utiliza mais fartamente sinais químicos para uma imensa gama de fins, incluindo reconhecimento, atração, afastamento e agregação de conspecíficos (WYATT, 2014). Quando tais sinais são utilizados com objetivos de comunicação entre indivíduos de uma mesma espécie, são denominados de feromônios (WYATT, 2010). No entanto, as características do meio e o uso concomitante de outros sentidos interferem na maneira como os feromônios são utilizados e cada táxon exibe estratégias particulares de emissão, recepção e resposta a estes sinais. A maioria das espécies de mariposas possui hábitos noturnos, portanto a luz se configura como recurso escasso e a sensibilidade a cores é radicalmente comprometida. A utilização da visão para primeiramente localizar e depois diferenciar potenciais parceiros faz pouco sentido nesse tipo de cenário. As espécies noturnas contornaram essa aparente desvantagem investindo fortemente na produção de feromônios pelas fêmeas (ALLISON; CARDÉ, 2016) e na captação de voláteis por parte dos machos com o desenvolvimento de antenas hipersensíveis (SCHNEIDER, 1964). De fato, o primeiro feromônio a ser isolado foi extraído de fêmeas de *Bombyx mori* (Saturniidae), o que determinou sua denominação como bombicol (BUTENANDT et al., 1959).

A adoção de hábitos majoritariamente diurnos pelas borboletas, por outro lado, abriu um leque de recursos completamente novo e possibilitou o desenvolvimento e seleção de estratégias fisiológicas, comportamentais, morfológicas e ecológicas bastante diversas àquelas usualmente encontradas na maioria das espécies de lepidópteros noturnos. A localização e atração de parceiros quando há iluminação suficiente possibilita ampla sinalização visual, especialmente através do uso de padrões complexos de cores que envolvem intensa pigmentação, coloração física por refração e sensibilidade à luz ultravioleta (SILBERGLIED; TAYLOR, 1973; JIGGINS et al. 2001; PAPKE et al., 2007; MÉROT et al., 2015). Padrões de coloração tão sofisticados e antenas aparentemente pouco desenvolvidas em comparação àquelas das mariposas, poderia fazer crer que a utilização de sinais químicos por borboletas desempenharia um papel secundário. No entanto, a observação de que agrupamentos de escamas especializadas ocorriam em asas de machos de diversas espécies de borboletas feita por SCUDDER (1877) e concomitantemente por MÜLLER (1877), levantou a hipótese de que tais estruturas estariam envolvidos na emissão de odores, sendo provavelmente produtos de seleção sexual. A descoberta gradual de várias estruturas masculinas similares àquelas que fêmeas de mariposas usavam para disseminar odores endossou a hipótese de que as “máculas sexuais” denominadas por Müller estavam de fato envolvidas na estocagem e secreção de

feromônios (BARTH, 1960; VANE-WRIGHT, 1972). Essas estruturas foram denominadas de órgãos odoríferos, órgãos androconiais ou simplesmente, androcônias.

Estruturalmente, androcônias são aglomerados de escamas morfologicamente diferenciadas ou de pelos eversíveis e alongados. Estes últimos localizam-se ne região genital, associados às glândulas genitais, ou nas asas, protegidos por dobras na membrana (VANE-WRIGHT; BOPPRÉ, 1993). Manchas androconiais localizam-se em praticamente todo o corpo, mas são mais comuns nas asas e abdome, com formas e arranjos intermediários (VANE-WRIGHT, 1972; SCOBLE, 1992; HALL; HARVEY, 2002). As escamas e pelos androconiais por sua vez, também exibem uma miríade de formas e micro estruturação (DOWNEY; ALLYN, 1975; HALL; HARVEY, 2002; HERNÁNDEZ-ROLDÁN; BOFILL; DAPPORTO, 2024, DARRAGH et al., 2017).

Talvez pela maior facilidade de obtenção de quantidades mínimas necessárias para análise química dos compostos e certamente pela sua importância econômica devido a ampla ocorrência de espécies-praga, os esforços e recursos tradicionalmente priorizaram estudos com feromônios de mariposas. O desenvolvimento posterior de técnicas analíticas mais sensíveis tornou possível a investigação daqueles compostos envolvidos na comunicação de borboletas. O primeiro estudo que isolou compostos químicos em borboletas foi publicado por MEINWALD et al. (1969), os quais identificaram alcalóides pirrolizidínicos e ésteres alifáticos provenientes de glândulas abdominais de *Lycorea* (Nymphalidae: Danaini). A hipótese de que as secreções atuariam como afrodisíacos foi novamente levantada e tais conclusões foram investigadas mais a fundo em estudos quase simultâneos de PLISKE; EISNER (1969) e MYERS; BROWER (1969). Sucederam-se então as primeiras publicações em que se isolaram compostos provenientes de androcônias alares de borboletas: BERGSTROM; LUNDGREN (1973) que identificaram neral, geranial e geraniol em *Pieris napi* (Pieridae: Pierinae) e LUNDGREN; BERGSTROM (1975), isolando nonanal e acetato de hexadecil em *Plebejus argyrognomon* (Lycaenidae: Polyommatinae).

A abordagem bioquímica em estudos subsequentes possibilitou o acúmulo de evidências que apontaram para um papel complementar dos estímulos químicos frente aos visuais. Para borboletas, atualmente, tem-se como consenso de que sinais visuais são preponderantes em distâncias maiores – quando ocorre a localização de potenciais parceiros – e que sinais químicos passam a atuar a partir da aproximação dos indivíduos. Em curtas distâncias, portanto, a comunicação química atua complementarmente à visual e a importância relativa de cada estímulo depende da espécie ou grupo envolvido (CONSTANZO; MONTEIRO, 2007; PAPKE et al., 2007; ESTRADA; JIGGINS, 2008; KEMP; RUTOWSKI, 2011; MÉROT et al., 2015;

BACQUET et al., 2017). Dessa forma, compostos masculinos androconiais de fato atuam na comunicação interespecífica de borboletas e podem ter natureza sexual (GRULA et al., 1980; SILBERGLIED 1984; VANE-WRIGHT; BOPPRÉ 1993; COSTANZO; MONTEIRO, 2007).

Feromônios sexuais desempenham papel importante sobre o sucesso reprodutivo dos indivíduos e como são espécie-específicos por natureza (WYATT, 2014), atuam fortemente no isolamento reprodutivo entre espécies (JOHANSSON; JONES 2007; SMADJA; BUTLIN, 2008). Com relação a borboletas, até recentemente estudos a respeito da própria composição química de feromônios masculinos e do seu papel na comunicação e isolamento reprodutivo concentraram-se em espécies Neárticas de Pieridae (BERGSTRÖM; LUNDGREN, 1973; GRULA et al., 1980, ANDERSSON et al., 2007), do gênero *Biclyclus* africanas (Nymphalidae) (COSTANZO; MONTEIRO, 2007, NIEBERDING et al., 2008; BACQUET et al., 2015) e de espécies africanas e americanas da subfamília Danainae (Nymphalidae) (MEINWALD et al., 1969; PLISKE; EINSNER, 1969; BOPPRÉ, 1984; VANE-WRIGHT; BOPPRÉ, 1993). Para borboletas Neotropicais, os esforços estavam voltados ao estudo de compostos derivados de alcalóides pirrolizidínicos usados por Danainae (BOPPRÉ, 1984; TRIGO et al., 1996a e 1996b e referências contidas; SCHULZ et al., 2004).

A partir da década de 2010, floresceu grande interesse em aspectos da ecologia química de Heliconiini e Ithomiini (Nymphalidae). Esforços vem sendo direcionados à identificação e descrição de seus compostos androconiais e investigação de sua influência sobre comportamento reprodutivo e evolução (MÉROT et al., 2015; MANN et al., 2017, DARRAGH et al., 2017, 2019, 2020). Heliconíneos e Itomíneos compreendem os grupos de borboletas que participam dos complexos miméticos mais requintados do reino animal. Acredita-se que a comunicação sexual, pelo menos em *Heliconius*, depende fortemente de pistas visuais e machos preferencialmente cortejam fêmeas que tenham padrão de cor similar ao seu (JIGGINS et al., 2001; KRONFORST et al., 2006; MERRILL et al., 2011). Embora usualmente um mesmo anel mimético não contenha espécies que tenham grande proximidade filogenética, existem casos que incluem espécies irmãs, como ocorre entre *H. melpomene* e *H. timareta* (MÉROT et al., 2013). Em cenários como esse, a similaridade morfológica por si não impede o isolamento reprodutivo e feromônios passam a desempenhar papel de maior importância no reconhecimento e atração de pares (MÉROT et al., 2015; DARRAGH et al., 2017).

Existe grande biodiversidade de Heliconiini e Ithomiini na floresta Atlântica brasileira, onde várias espécies são tradicionalmente divididas em raças geográficas ou subespécies, relativas a diferentes subregiões (BROWN, 1975; 1977; LAMAS, 2004). Epítetos infraespecíficos são amplamente aplicados a borboletas, uma vez que o uso do conceito de

subespécies possibilita a associação de informações biogeográficas a diferentes grupos de populações (LAMAS, 2004). No entanto, seu uso é controverso e a validade científica deve ser testada por diferentes abordagens. São abundantes os casos de hibridização intra e interespecífica relatados para itomíneos e heliconíneos (D'ALMEIDA, 1951; BROWN, 1977; BROWN, 1982), com consequências diversas, desde inviabilidade reprodutiva, introgressão, aumento de pressão de predação ou simplesmente dispêndio energético (MALLET et al., 2007; ARIAS et al., 2008; GARZÓN-ORDUÑA; BROWER, 2017; MASSARDO et al., 2020).

A formulação do projeto desta tese de doutorado levou em consideração a existência de grandes lacunas químicas sobre a comunicação química em borboletas neotropicais, assim como questionamentos de natureza biogeográfica. A existências várias espécies de borboletas com populações morfologicamente distintas, limitadas a sub-regiões da floresta Atlântica, que possuem histórias biogeográficas distintas, torna a região ideal para comparações com o grupo. Finalmente, o uso de atributos diretamente relacionados à reprodução, como é o caso dos feromônios sexuais e a relativa novidade do tema justificaram a formulação dos objetivos da desta tese, aqui apresentados.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Investigar a composição química de secreções androconiais de borboletas do clado *Colias* (Pieridae: Coliadinae) e das tribos Ithomiini e Heliconiini (Nymphalidae: Danainae e Heliconiinae), relacionando-a a aspectos de sua morfologia alar, taxonomia e diversidade em diferentes sub-regiões da floresta atlântica brasileira.

1.2.2 Objetivos específicos

- Isolar os compostos químicos de secreções androconiais alares de borboletas Ithomiini, Heliconiini e do clado *Colias*.
- Comparar a composição química androconial entre espécies e entre categorias taxonômicas infraespecíficas de borboletas Ithomiini, Heliconiini e do clado *Colias*.
- Avaliar o efeito de centros de endemismo e áreas relativas a refúgios pleistocênicos da floresta Atlântica brasileira sobre a composição química androconial de borboletas Heliconiini e Ithomiini

- Avaliar possível relação entre distância geográfica e similaridade química dos compostos androconiais de borboletas Heliconiini e Ithomiini na floresta Atlântica brasileira
- Determinar relação entre distância genética e similaridade química dos compostos androconiais de borboletas do clado *Colias*

Os resultados desta tese estão divididos em duas partes. A primeira parte refere-se ao **Artigo I que** tratou da investigação da química androconial de cinco espécies simpátricas de borboletas do clado *Colias*, da descrição de suas escamas androconiais alares e da investigação da influência genética sobre a similaridade da composição química. Na segunda parte, o **Artigo II**, se investigou a composição química androconial de borboletas Ithomiini e Heliconiini em sub-regiões da floresta Atlântica, comparando-a entre categorias taxonômicas e entre unidades biogeográficas. Finalmente, o **Apêndice** contém o artigo referente ao **Artigo I**, recém-publicado no periódico *Organisms Diversity & Evolution*.

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2 RESULTADOS

Os resultados desta tese estão contidos em dois artigos científicos, que se seguem.

2.1 ARTIGO I: SPECIALIZED ANDROCONIAL SCALES CONCEAL SPECIES-SPECIFIC SEMIOCHEMICALS OF SYMPATRIC SULPHUR BUTTERFLIES (LEPIDOPTERA: PIERIDAE: COLIADINAE)

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Abstract

Chemical cues play an important role in short-range communication of butterflies, remarkably in sexual attraction and mate choice. Differentiated scale patches on the wings of male butterflies, the androconia, are involved in the emission of pheromones. Here we describe the androconial morphology of six sympatric species of Neotropical sulphur butterflies belonging to two genera of the *Colias*-clade (Pieridae) based on SEM imaging. Gas chromatography-mass spectrometry analyses were used to access the chemical compositions of androconial secretions, which were comparatively investigated to determine species-specific trends and to verify if they yield a phylogenetic signal. The androconial patches from all species are differentiated from the non-androconial male wing surface and exhibit morphological features that may act in both preventing the volatilization of secretions and facilitating the release of semiochemicals, such as high density and length of scales and large perforations in the upper lamellae. A total of 55 compounds were exclusive to the androconia and unique chemical profiles are present in each butterfly species, verified through multivariate analysis. The majority of androconial compounds were autapomorphic for each species and only four were dominant in more than one species. Cluster analyses placed the two species of *Anteos* in a single clade, but otherwise evidenced low similarities in the androconial secretion compositions among species, and a weak correlation between genetic distances and chemical dissimilarities was obtained. Our findings suggest that androconial substances are involved in mating oriented strategies and might be associated with the evolutionary history of the reproductive isolation of sulphurs.

Key words: Butterfly communication, Papilionoidea, *Phoebis*, phylogenetic signal, sex pheromones, sex-related traits

Introduction

Visual and chemical cues play complementary roles in the sexual behaviour of butterflies (Constanzo and Monteiro 2007; Smadja and Butlin 2009). While visual cues are involved in both long and short-range intraspecific interactions, chemical cues are preponderant at close interactions among the Papilionoidea (Grula and Taylor 1980; Silberglied 1984; Vane-Wright and Boppré 1993). The relative importance of each stimulus on butterfly mate recognition and mate choice may vary according to species (Papke et al. 2007; Constanzo and Monteiro 2007), but this multimodal signalling plays a pivotal role in pre-zygotic reproductive isolation among sympatric closely-related species (Bacquet et al. 2015). Thus, it is assumed that at least one of these components (e.g. color pattern, UV reflection, pheromones) is species-specific.

Some Coliadinae butterflies (Lepidoptera: Pieridae) are given the vernacular denomination of ‘sulphurs’, which is associated to the bright white/yellowish color of their wings from the deposition of pterin pigments on the scales (Watt 1964). In northeastern Brazil there are six sympatric species of sulphur butterflies belonging to the monophyletic *Colias*–clade (*sensu* Wahlberg et al. 2014), among which males are readily distinguished from one another by wing color pattern. Since visual stimuli play a major role in conspecific recognition of butterflies, including Pieridae – revised on Kemp and Rutowski (2011) –, a first glance assumption can be that the pronounced differences in wing color patterns would suffice for females to recognize conspecific males. However, males of all six species have differentiated, sex-specific scale patches on their wings – the androconia. These structures are involved in the emission of chemical compounds exclusive to male lepidopterans – especially butterflies and diurnal moths – which function as aphrodisiacs (Pliske and Eisner 1969; Nieberding et al. 2008; Yildizhan et al. 2009) or anti-aphrodisiacs (Estrada et al. 2011). Size, number and location of the androconia vary depending on the species, and their morphology appears to be related to the storage and discharge of pheromones produced by the secretory cells which are usually located at the base of the androconia (Kristensen and Simonsen 2003). For example, during courtship and for aggregation purposes, male Ithomiini butterflies (Nymphalidae, Danainae) display otherwise concealed erectile alar fringes involved in the dispersion of volatile compounds (Shulz et al. 2004). The androconia of male pierid butterflies are typically arranged in dense clusters of scales (Barth 1960) that do not have any protection to prevent evaporation of secretions besides the overlapping of the wings, when at rest. Maybe because of this lack of specialization in concealment, highly volatile androconial compounds would be disadvantageous and, in fact, the androconial chemical bouquets of pierids such as *Colias* (Coliadinae) may present large amounts of heavier and less volatile compounds (Grula et al. 1980). On the other hand, compounds with higher volatility, such as geranial and neral, occur in high concentrations in *Pieris* (Pierinae) and are released mainly during flight activity (Andersson et al. 2007).

Results are contrasting regarding a phylogenetic signal of androconial secretions in different groups of butterflies. When the chemical compounds of sympatric, mimetic species were analysed, limited phylogenetic signal was found within both Ithomiini (Schulz et al. 2004) and Heliconiini (Mann et al. 2017) (Nymphalidae: Danainae and Heliconiinae, respectively). A sophisticated convergent aposematism (e.g. bright color, high-contrast patterns) might cause confusion in intraspecific recognition, as observed in *Heliconius* (Estrada and Jiggins 2008). Thus, it is reasonable to assume that in order to avoid hybridization, distinct chemical signatures must be adopted between closely related comimetics (Mérot et al. 2015). Nonetheless,

androconial secretions of mimetic milkweed butterflies (Nymphalidae: Danainae) exhibit strong phylogenetic chemical signalling in the species level (Schulz et al. 1993). In the same way, a strong correlation was demonstrated to occur between secretions of androconia and genetic distances of partially sympatric species of *Pyrgus* (Hesperiidae: Pyrginae) (Hernández-Roldan et al. 2014). Species of this genus are morphologically very similar but do not rely on mimicry.

In this study, we present a different scenario, in which six closely related, apparently non-mimetic species belonging to the *Colias*-clade co-occur. We aimed to: 1) describe the morphology of the androconia of the six species and access the biological significance of key features; 2) investigate whether there are chemical compounds exclusive to the androconial patches in relation to the remaining non-androconial wing surface and to those of the wings of conspecific females, and 3) compare the composition of androconial secretions among the six species and verify whether there is phylogenetic signal for this trait.

Materials and Methods

Studied butterflies

We investigated the sulphurs *Anteos clorinde* (Godart, [1824]), *A. menippe* (Hübner, [1818]), *Phoebis argante* (Fabricius, 1775), *P. philea* (Linnaeus, 1763), *P. marcellina* (Cramer, 1777) and *Phoebis statira* (Cramer, 1777) (Figure 1). We followed the classification of Murillo-Ramos et al. (2018), who proposed *Aphrissa* as a synonym of *Phoebis*, and of Núñez et al. (2020), who raised *P. marcellina* to the species rank. All these species are typical of sunny, open areas, sympatric and to some extent, synchronic in northeastern Brazil in the same manner to what happens on most of their range (DeVries 1987; Brown 1992).

Scanning electron microscope (SEM) imaging and description of androconial scales

The androconial patches and surrounding wing areas of dry mounted males belonging to the six studied species were excised from the wings using surgical scissors which were rinsed with hexane and dried between uses. The samples were placed in aluminium stubs with double-sided carbon adhesive tape and coated with 80% gold / 20% palladium in a Jeol Datum Ion Sputter JFC-1100. The metalized samples were examined in a Zeiss EVO LS15 scanning electron microscope and images were obtained at an electron high tension of 10 kV. The density of scales was estimated by counting one quadrant of a 1 mm^2 area and multiplying the value by four. The length of the scales (both androconial and ordinary) was measured from base to apex for each species (mean ± Standard Deviation; n = 10 per species).

The overall shape, perforations (windows) and density of the scales surrounding the androconial patches varies noticeably, depending on the area of the wing they cover. Therefore, for comparative purposes, we considered only the ordinary scales laterally adjacent to the androconia. To describe the scales, we followed Downey and Allyn (1975) with adaptations. The nomenclature for the microstructures of the scales follows Ghiradella (1989). For the wing areas and venation nomenclature, we followed DeVries (1987).

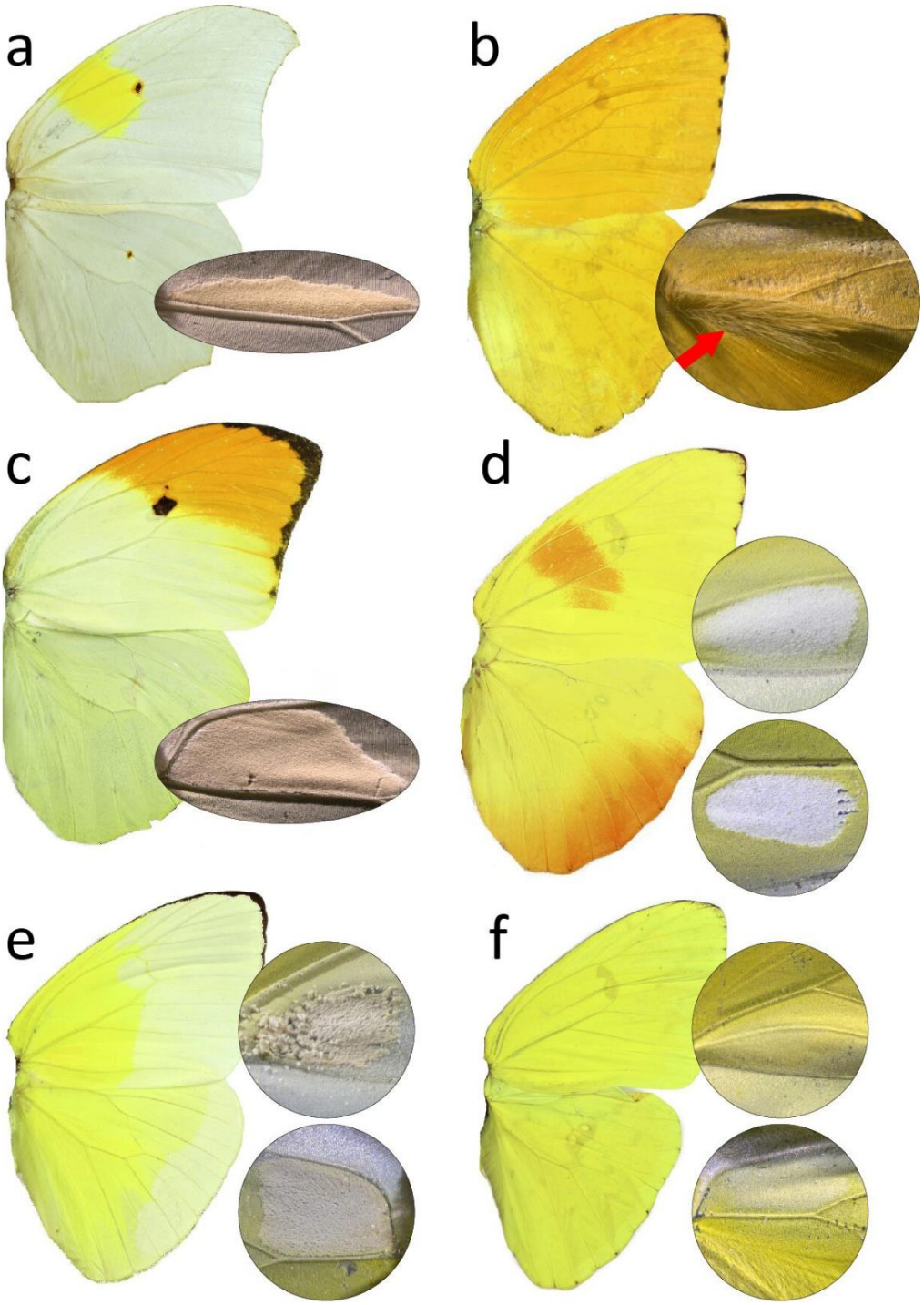

Figure 1. Male dorsal wing colour patterns of the Colias-clade butterflies of northeastern Brazil: (a) Anteos clorinde, (b) A. menippe, (c) Phoebis argante (d) P. philea, (e) P. statira, and (f) P. marcellina. Details of the

androconia (circles to the right), showing the shapes and colour distinction from the surrounding scales. The red arrow indicates a tuft of hair-like scales below the androconial patch.

Sampling and chemical analyses of androconial secretions

Butterflies were sampled in the municipalities of Recife (8° 03' 10''S, 34° 56' 51''W) and Buíque (8º 35' 11''S, 37° 08' 52'' W), state of Pernambuco, northeastern Brazil. All individuals were captured *in situ* visiting flowers of *Ixora coccinea* L. (Rubiaceae) or *Bougainvillea spectabilis* Willd. (Nyctaginaceae) from 09:00h to 13:00h.

The methodology of sampling, injection and chemical analyses of the androconial secretions was adapted from Mann et al. (2017). Immediately following capture, the individuals were sacrificed by thorax compression while inside the insect net. Androconial patches on the wings of each male (n = 5 – 12 / species) were excised and inserted into clear 2 ml silanized glass vials containing hexane (≥ 99.7% purity, Sigma–Aldrich, USA; bidistilled prior to use). In order to latter exclude from our analyses the chemical compounds not exclusive to the androconia, the same procedure was applied to equivalent excised wing areas from conspecific females (n = 2 / species) and to non-androconial male wing areas (n = 1 / species). In all cases, the butterflies were manipulated by the thorax to avoid contamination of the wings. Solvent negative controls were obtained for each sampling event (n = 6). The crude solvent extracts were filtered through a silanized glass Pasteur pipette plugged with silanized glass wool (Sigma-Aldrich, USA) to remove floating scales and other debris, then concentrated to approximately 25 µl under a laminar N_2 flow and kept at -24° C refrigeration until further processing procedures.

For gas chromatography-mass spectrometry (GC-MS) analyses, we used a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent 7890A™ gas chromatograph, Agilent 5975C Series MSD™ mass spectrometer) and equipped with a non-polar HP-5ms column (Agilent J&W; 30 m × 0.25 mm d.i., 0.25 µm film thickness). A split/splitless inlet was fitted with an Agilent Thermal Separation Probe (TSP). For each sample, 1 µl of the eluate was injected into quartz microvials which were then inserted in the TSP vial holder with the inlet set to split mode (1:1) and the injector temperature set to 250 °C. GC oven temperature was set at 60 °C for 2 min and then increased at a rate of 10 °C/min^{-1} to 280 °C, which was held steady for 12 min. The carrier gas flow was maintained at a constant pressure of 7.3 psi. MS Source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Mass spectra were taken at 70 eV in EI mode with a scanning speed of 1.0 scan^{-s} from *m/z* 35–450.

After the chromatograms were obtained, their peak areas were integrated by using the software MSD ChemStation E.02.01.1177 (Agilent Technologies, Palo Alto, USA) to obtain the total ion current signal. A homologous series of linear alkanes (C_8 – C_{40}) was used to determine linear retention indices (RI) of the compounds (Van den Dool and Kratz 1963), which were then tentatively identified by comparing their mass spectra and retention indices with those of reference samples available from personal and commercial mass spectral libraries (FFNSC 2, MassFinder 4, NIST14 and Wiley Registry™ 9th ed.). Mass spectra of target compounds and their calculated retention indices were also cross referenced with data published by Hayashi et al. (1977), Grula et al. (1980), Honda and Kawatoko (1982) and Yildizhan et al. (2009). Additionally, the authors consulted mass spectrometry specialist Prof. Dr. Stefan Schulz (Technische Universität Braunschweig, Germany) for input on identifications. We only considered peaks exclusive to the androconial extracts and that were present in more than two individuals, which were used to determine the relative percentages of each compound per sample. In that way, some compounds such as the cuticular hydrocarbon 13-methylheptacosane – an important male pheromone of the sulphur *Colias philodice* Godart, 1819 (Grula et al. 1980) – were excluded from our characterization of androconial secretions because they were present in female or non-androconial male wing extracts as well. For comparative analyses of androconial extracts, we established as a threshold that compounds should be present at over 1% of the total individual peak area in at least one of the analysed samples for any species. Compounds with relative concentrations higher than 10% of total peak area were classified as 'dominant', whereas 'minor compounds' were present at concentrations between 1% and 10%. We considered compounds that eluted latter than pentacosane (RI = 2500) as lowly volatile (Table 2), according to Mann et al. (2017), which also worked on a non-polar column (HP-5ms). Non-polar GC columns are made with poorly selective stationary phases, and thus commonly applied to separate non-polar compounds roughly based on volatility.

Statistical analyses

The presence/absence of componds and their relative percentages were used to generate Jaccard- and Bray Curtis-based similarity matrices respectively, from which non-metric multidimensional scaling analyses were performed in PAST 4.02 (Hammer et al. 2001). To verify if the chemical profile within each category (i.e., species) was significantly different, ANOSIM analyses wereperformed. In order to avoid bias from excessive weight of major compounds, their relative amounts were submitted to a *a priori* square root transformation.

To verify whether the results reflect the phylogenetic arrangement of the studied species, resulting dendrograms were compared to the most recent phylogeny of the group (Murillo-Ramos et al. 2018) and a Mantel test was performed to correlate the matrices of chemical dissimilarity and genetic distance using the packages Vegan (Oksanen et al. 2013) and Ade4 (Dray and Dufour 2007) in R software (R Core Team, 2016). The genetic distance matrix was built by calculating pairwise distances of the mitochondrial cytochrome C oxidase 1 (COI) gene using the Kimura 2-parameter (K2P) model (Kimura 1980), with the data available from the Barcode of Life Data System (BOLD) platform (Ratnasingham and Hebert 2007) (Supplementary table S1) with MEGA-X 10.1 (Kumar et al. 2018). We excluded *A. menippe* from this analysis because genetic data is not available for this species.

Results

Morphology of androconial scales

The androconia of the forewings are always on the ventral side while those of the hindwings are dorsally positioned (Table 1). Unless specified otherwise in Table 1, the scale sockets are always visible, pedicels are positioned at the basis of the median line, and the longitudinal ridges on the lamina are parallel and straight (Figure 2). The color of androconial scales are always paler than that of the surrounding ordinary scales (Figure 1) and contrarily to the latter, the morphology of androconial scales is relatively constant intraspecifically. Androconial scales are 1.5 to 4 times more densely arranged in the wing surface than the ordinary scales (Table 1, Figure 2) and inserted in the wing membrane at wider angles (not measured). The windows (perforations) and the respective crossribs on the upper lamella are absent or less evident in the periphery of the scales and are usually less evenly arranged than that of the ordinary scales (Table 1, Figure 2).

Androconial secretions chemistry

A total of 55 compounds were found exclusively in the androconial extracts of the six species, of which 41 were autapomorphic (Table 2, Supplementary table S2). The most complex androconial chemical profile was obtained from *Phoebis philea*, with the highest number of both overall and species-exclusive compounds (23 and 14, respectively). It also presented the highest number of compounds with high RI's (suggesting lower volatility), and it is in this amplitude that the most dominant constituents appear (Table 2; Supplementary table S2). This also occurred in *P. statira*, but the profiles of the androconial extracts of the two other

congenerics are rather distinct. Only a few compounds identified in *P. argante* and *P. marcellina* have high RI's, whereas the most dominant ones are much more volatile: in *P. marcellina*, benzyl salicylate, an unassigned hexadecenoic acid, and hexadecanoic acid and in *P. argante*, 6,10,14-trimethyl-pentadecan-2-ol, which comprises ca. 90% of the total blend (Table 2). Androconial solvent extracts of *P. argante* contained the least number of compounds (6) and most restricted molecular weight amplitude (Supplementary table S2); nonetheless, as only five samples were analysed, it is plausible that this species was under sampled.

As a general observation, the chemical blends of androconial extracts from all species revealed few dominant compounds: two or three compounds corresponded to over 50% of the total blend, while a wider array of compounds occurred in lower concentrations (Table 2; Supplementary table S2).

Table 1. Morphological aspects of androconial and surrounding ordinary scales of the *Colias*-clade butterflies of northeastern Brazil.

Species	Location of the Androconia	Scales										
		Androconial						Ordinary				
		Density (mm^2)	Lenght (µm)		Shape	Upper Lamina		Density (mm^2)	Lenght (µm)		Shape	Upper Lamina	
			Averaged	SD		Windows	Crossribs		Averaged	SD		Windows	Crossribs
***Anteos clorinde*†**	HW: Dorsally, in Sc+R_1 from post-basal to medial area	850	207.6	22.5	Oblong with rounded apex and obtuse base	Irregularly sized	Unevenly spaced	220	116.5	5.5	Obovate with truncate apex and auriculate base	Regularly sized	Evenly spaced
Anteos menippe	HW: Dorsally, in Sc+R_1 from post-basal to sub-medial area	600	198.4	8.8	Oblong with rounded apex and obtuse base	Regularly sized, rounded	Evenly spaced	270	113.9	6.7	Obovate with truncate apex and auriculate base	Regularly sized	Evenly spaced
Phoebis argante	HW: Dorsally, on the submedial area of Sc+R_1 and Rs.	680	Not measured		Obovate with rounded apex and auriculate base	Very small, regularly sized	Evenly spaced	300	86.1	3.1	Obovate with rounded or truncate apex and auriculate base	Absent	Absent
Phoebis philea	FW: Ventrally, in CuA_2, on submedial area HW: Dorsally, in Sc+R_1 from post-basal to submedial area	650	127.7	13.3	Oblong with slightly rounded apex and obtuse base	Regularly sized on the distal half. Considerably larger and irregularly sized on the proximal half	Unevenly spaced, much more prominent on the proximal half	350	94.3	2.5	Oblong with truncate apex and auriculate base	Regularly sized	Evenly spaced
Phoebis marcellina	FW: Ventrally, in CuA_2 from post-basal to submedial area HW: Dorsally, in Sc+R_1 from post-basal to submedial area	570	81.9	4.5	Oblong with rounded apex and obtuse base	Regularly sized	Evenly spaced	380	84.8	3.6	Oblong, but wider than the androconial ones, with truncate apex and auriculate base	Absent	Absent
Phoebis statira	FW: Ventrally in CuA_2 from post-basal to	650	117.5	4.6	Ovate, with distal 1/5 abruptly straightening towards the apex,	Variable length, the ones on the centre may reach approximately	Unevenly spaced	300	80.9	8.5	Ovoid with asymmetrical margins and pointy apex. Pedicel dislocated to	Very small, rounded	Rudimentary or absent. Ridges oblique and slightly curved

	sub-medial area	which is acute. Truncate base	1/2 the total length of the scale				the right of median line		
	HW: Dorsally, in Sc+R_1 from post-basal to sub-medial area			400	86.5 / 60.1	7.7 / 3.5	Obovate with truncate apex and obtuse base	Regularly sized	Evenly spaced

†Socket of the androconial scales are involved by a swollen basal area of the scale, which covers the pedicel in lateral view.

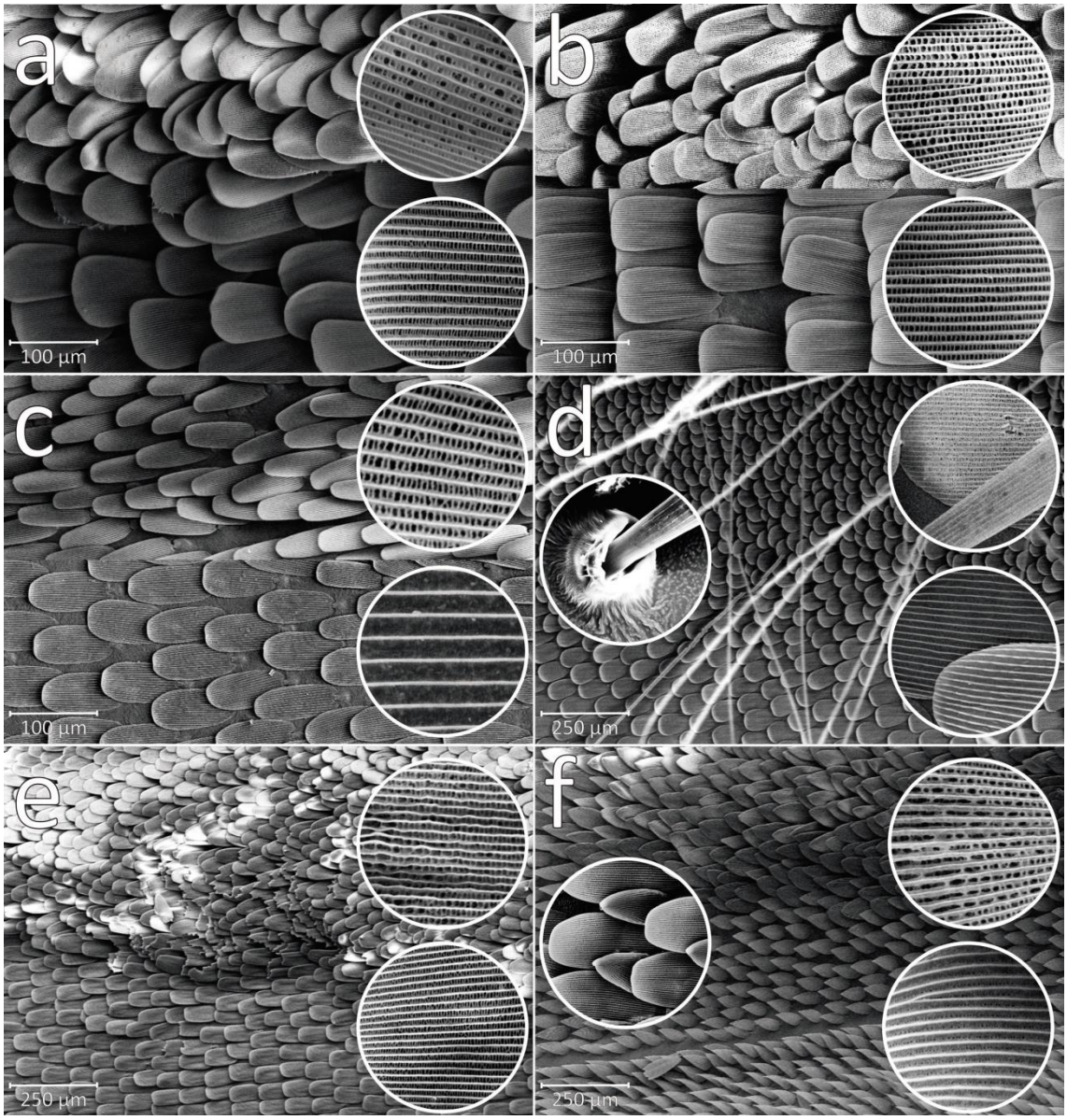

Figure 2. SEM photographs of wing scales of the *Colias*-clade butterflies of northeastern Brazil. The androconial scales are on the upper portion of the images and the ordinary scales, on the lower portion. At 500x magnification: (a) *Anteos clorinde*, (b) *A. menippe*, (c) *Phoebis marcellina; at 200x magnification:* (d) *P. argante*, (e) *P. philea*, (f) *P. statira*. Details of the scales (circles to the right), showing the striae, crossribs and windows are at 2.5k magnification, with exception of (c) and (e), at 5k magnification. Details on circles to the left: (d) differentiated socket of hair at 5k magnification and (f) dissimilar shapes of ordinary scales at 1.5k magnification.

Although there were slight intraspecific variations in the androconial chemical composition, each species exhibited a characteristic chemical profile ($p = 0.001$, Global-R = 0.999, Bray Curtis-based; $p = 0.001$, Global-R = 1, Jaccard-based) and the NMDS ordination segregated the samples (or individuals) in a strongly grouped distribution, relative to species (Figure 3, Supplementary figure S1). Some compounds were found in androconial extracts of

more than one species, but only four were dominant (≥ 10% / species) across species: benzyl salicylate in *A. menippe* and *P. marcellina*; hexadecanoic acid in *P. statira* and *P. marcellina*; an unidentified compound (unassigned compound 5) in *P. statira* and *P. philea* and an unidentified aliphatic ester (unid. aliphatic ester 5) in both species of *Anteos* (Table 2). These co-occurrences of dominant compounds heavily influenced the clustering pattern, as evidenced by the comparison of presence/absence to the relative abundances data (Supplementary figure S2). The Bray Curtis analysis yielded higher similarities among males of the two species of *Anteos*; the males of *A. clorinde* did not even form a cluster of their own, but were rather clustered together with *A. menippe* (Figure 4, Supplementary figure S2 - A). Furthermore, higher similarities were obtained between *P. marcellina* and *Anteos* spp. than between the former and its congenerics (Figure 4). Although some dominant compounds co-occurred in two, sometimes three of the investigated species of *Phoebis* (Table 2, Supplementary table S1), no androconial chemical synapomorphy was identified for the genus as a whole. The four species in our study presented very low similarity values, and the chemical blend of *P. argante* actually only shared a single minor compound – linoleic acid – with congenerics (Table 2; Supplementary table S2). As result, all samples of *P. argante* formed an outgroup distant from the remainder sulphurs (Figure 4, Supplementary figure S1). The Mantel tests revealed moderately positive correlations between genetic distances and chemical dissimilarities of the group (r = 0,593, p = 0.001, Bray Curtis-based; r = 0.509, p = 0.001, Jaccard-based) (Figure 5, Supplementary figure S3).

Table 2. Total relative amounts of dominant compounds (≥ 10% relative percentage in any sample) identified in androconial extracts of males of the *Colias*-clade butterflies (genera *Anteos* and *Phoebis*) of northeastern Brazil. Values between brackets indicate the number of analysed samples in which each compound was present, per investigated species.

Kovats Index	**Compound**	***A. menippe* (N = 10)**	***A. clorinde* (N= 8)**	***P. statira* (N=9)**	***P. marcellina* (N=10)**	***P. philea* (N=12)**	***P. argante* (N=5)**
1853	6,10,14-Trimethyl-pentadecan-2-ol	-	-	-	-	-	88.96 [5]
1892	Benzyl salicylate	9.94 [10]	-	0.45 [3]	37.12 [10]	-	-
1953	Hexadecenoic acid	1.61 [7]	-	0.37 [1]	49.14[10]	0.02 [1]	-
1961	Hexadecanoic acid	2.34 [8]	-	18.55 [8]	-	-	-
2470	Unid. aliphatic ester 2 *m/z*: 96,81,55,97,43	20.08 [10]	-	-	-	-	-
2476	Unassigned compound 1 *m/z*: 85,43,101,84,55	-	-	20.83 [9]	0.17 [2]	4.78 [12]	-
2532	13-Methylpentacosane	-	28.30 [8]	-	-	-	-
2582	Hexenyl octadecanoate	-	15.33 [8]	-	-	-	-

2674	Unid. aliphatic ester 5 *m/z*: 96,81,97,55,43	38.15 [10]	50.72 [7]	-	-	-	-
2691	2-Phenylethyl hexadecanoate	-	-	16.66 [9]	-	-	-
2956	Unassigned compound 5 *m/z*: 94,43,95,134,109	-	-	21.95 [8]	-	10.3 [12]	-
3156	Unassigned compound 9 *m/z*: 94,95,135,43,67	-	-	-	-	17.46 [11]	-
3176	Unassigned compound 11 *m/z*: 94,95,43,135,109	-	-	-	-	12.31 [11]	-
3197	Unassigned compound 12 *m/z*: 94,43,134,95,109	-	-	-	-	22.92 [11]	-

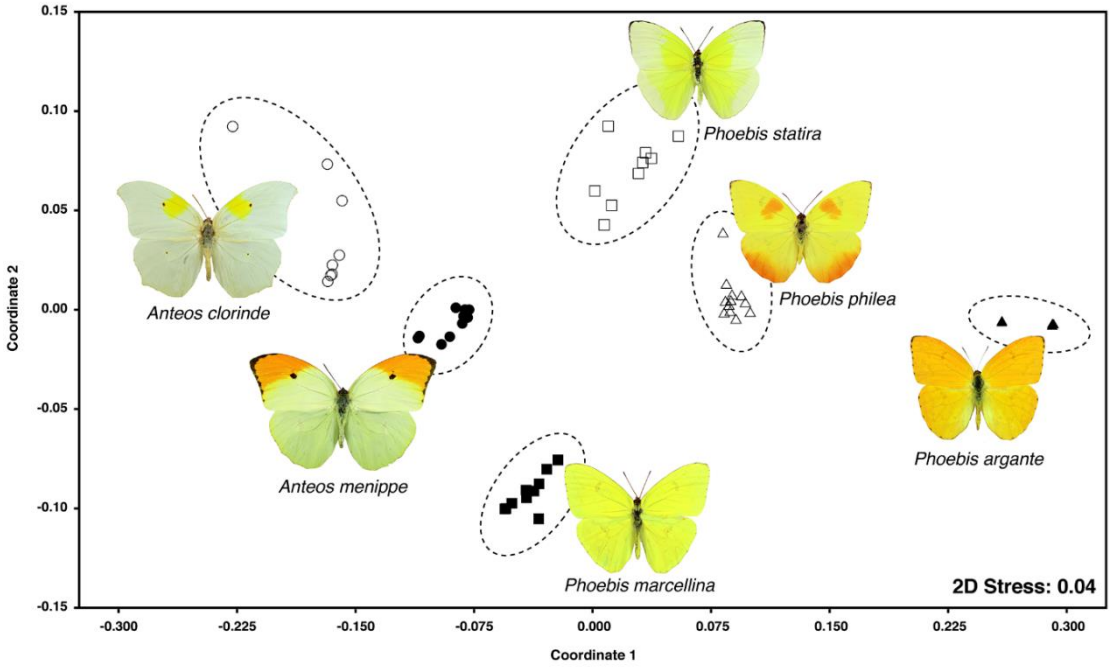

Figure 3. Bray Curtis-based NMDS ordination for the chemical composition of androconial secretions of the *Colias*-clade butterflies of northeastern Brazil.

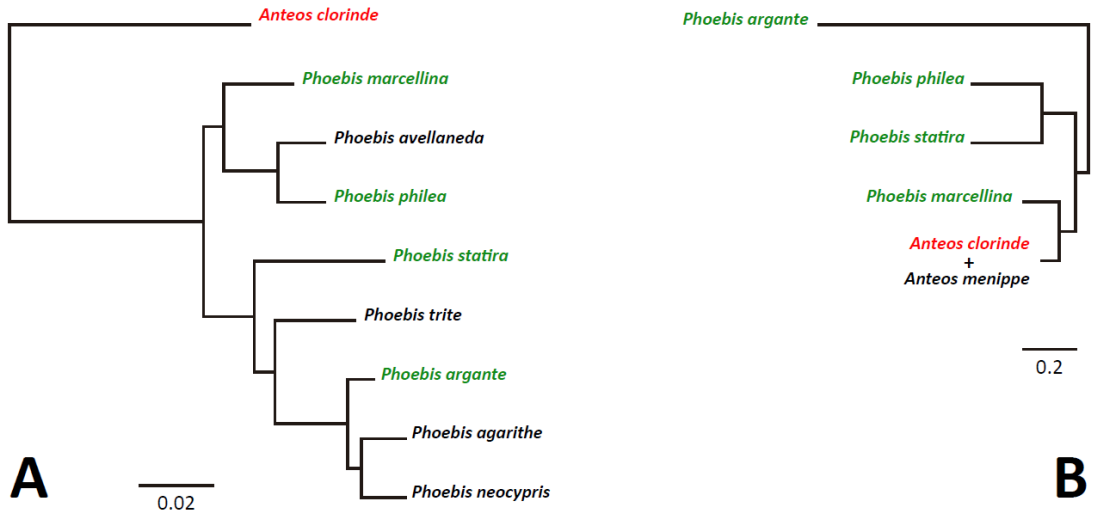

Figure 4. Comparison between the phylogenetic tree adapted from Murillo-Ramos et al. (2018) and the dendrogram based on the Bray Curtis similarity index from the androconial chemistry of the *Colias*-clade butterflies of northeastern Brazil.

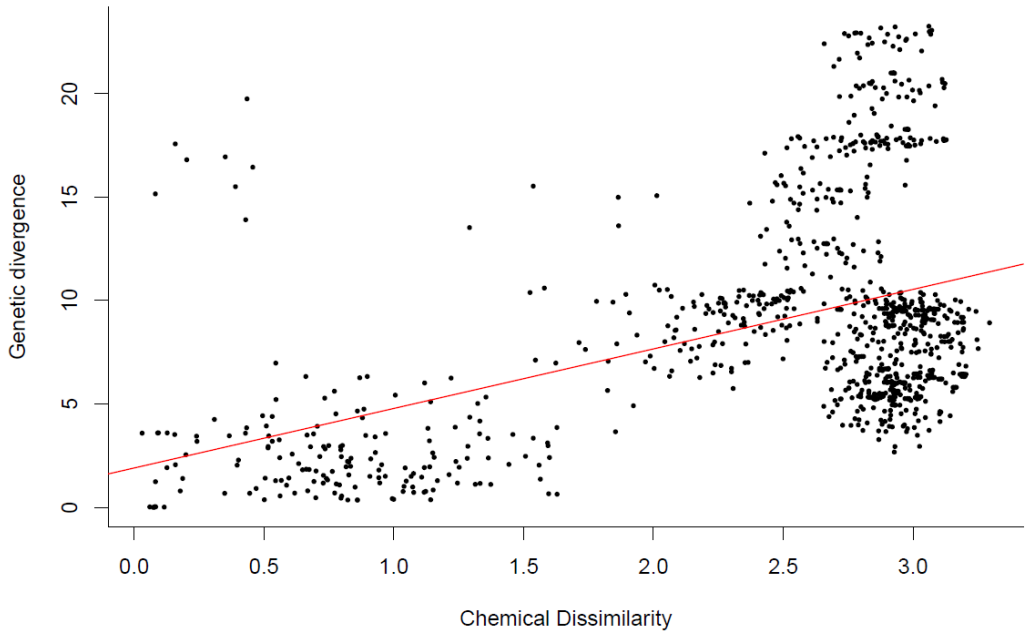

Figure 5. Correlation between genetic distance and Bray Curtis-based androconial chemical dissimilarity of the *Colias*-clade butterflies of northeastern Brazil.

Discussion

Morphology of androconial scales

The overall morphology of the wing scales of the six investigated species is typical of higher Lepidoptera: hollow structures that contain numerous striae and transversal crossribs that

delimit perforations along the upper lamella (Kristensen 1970). The androconial patches, however, are clearly differentiated from the typical scales of the surrounding wing areas by peculiar features such as paler color, higher density and length of scales and larger windows in the lamella. There is no apparent specialized structure on the wing membrane to protect those patches and reduce volatilization of secretions of the studied butterflies, such as the "pocket-like cavities" in the Danaini (Boppré 1993) and Ithomiini (Schulz et al. 2004). Nevertheless, the androconial patches of all species are invariably placed on the 'friction areas' of the wings, be it ventrally (post-basal to submedial region of CuA_2) or dorsally (post-basal to medial region of $Sc+R_1$). These areas remain overlapped when the animal is at rest, a mechanism suggested by Rutowsky (1980) as a mean of reducing the volatilization of androconial secretions in species of *Colias.* This indeed seems to be the case of the *Colias*-clade sulphurs, given that there are no androconial patches located in other areas of the wings.

Compared to ordinary scales, androconial scales of the *Colias*-clade butterflies were longer (with the exception of *P. marcellina* and *P. argante*), and more compactly packed, with densities 1.5 to 4 times higher than that of ordinary scales. Similar ratios (≈ 2 androconial: 1 ordinary) were observed in species of *Eurema* and *Colias* (Pieridae: Coliadinae) (Vetter and Rutowsky 1979), but not in *Pieris* (Pieridae: Pierinae), in which scent scales are widely distributed on the ventral surface of the wings, with a much lower density than that of ordinary scales (Yoshida et al. 2000). High density of scales may have a complementary effect to the abovementioned overlapping of the wings, as a preventive apparatus to the volatilization of secretions. In fact, most of the area occupied by androconial scales remain concealed by the upper and adjacent scales, with only the distal portions exposed.

The analysis of the micro sculpture patterns also revealed differences between androconial and ordinary scales. More prominent crossribs and larger windows occur in the upper lamella of the androconial scales, especially in the mid portion. The biological significance of the latter trait is not clear, but it may be related to a rapid release of male pheromones during courtship behaviour. According to Barth (1960), a suitable area in which volatilization of the secretion takes place is a distinguishing feature of androconial scales. Thus, by increasing the contact surface, the enlarged windows in a scale-dense region may contribute to the release of androconial secretions into the air or even facilitate the contact of less volatile compounds with the sensorial surface of conspecific female antennae.

What appeared to be a more specialized structure for releasing pheromonal secretions was found only on the androconia of *P. argante* males, in the form of a dense patch of long hair-like scales. Males of the remaning species lack those hairs on the androconial patches, but

present similar features ("fringes") on the anal margin of the forewings (Barth 1960; Bergström and Lundgren 1973), a "friction area" that is close to the ventral androconia and likely to rub into them during flight or courtship behaviour. Friction of the androconial patches by hovering or buffeting the wings near females is a widespread male behaviour among the Coliadinae (Rutowsky 1978; Silberglied and Taylor 1978; Vetter and Rutowsky 1979; Kan and Hidaka 1997). So, those fringes might have an analogous function to that of the long hair-like scales of *P. argante* and the potential to act as a facilitator for the release of androconial secretions. This could be considered as a composite system – an area where secretions are produced, associated to a structure that aids their release – as proposed by Barth (1960).

Androconial secretions chemistry

A wide array of compounds exclusive to androconial extracts were found for all investigated species. These chemicals occur precisely in the androconial patches described in this study, which demonstrates that, at least in the wings, their storage and release is restricted to these morphologically specialized areas. Also, we have shown that the composition of androconial secretions is species-specific, suggesting that these compounds take part in intraspecific chemical communication. Behavioural experiments have consistently demonstrated that certain male wing secretions function as sex pheromones in butterflies (Constanzo and Monteiro 2007; Darragh et al. 2017) including Pieridae (Taylor 1973; Silberglied and Taylor 1978; Grula et al. 1980; Rutowski 1980; Kan and Hidaka 1997; Andersson et al. 2007; Yildizhan et al. 2009). Although we did not conduct such experiments, the absence of androconial constituents from the wings of conspecific females offers compelling evidence of their involvement in mate recognition and/or attraction.

Our results revealed distinct chemical profiles for each investigated species of sulphur. Among butterflies, the degree of chemical differentiation of androconial profiles seems to vary case by case, as shown in studies with sympatric Nearctic and Palearctic Pieridae. *Colias philodice* and *C. eurytheme* Boisduval, 1852 present significant qualitative compositional differences (Grula et al. 1980), whereas for the closely related *Pieris melete* Ménétriés, 1857 and *P. napi* Linaeus, 1758, very similar androconial chemical profiles were evidenced. In this later case, marked dissimilarities were restricted to the concentrations of two stereoisomeric monoterpenes (neral and geranial) and a species-specific presence of another (linalool) (Hayashi et al. 1977). Among the species in our study, large qualitative and quantitative chemical differences were observed , which reinforces the highly species-specific character of the constituents in androconial secretions. This strong differentiation of sex-related traits, if

properly recognized by the females, may act alongside the also very distinct species-specific male color patterns, leading to a robust pre-zygotic isolation mechanism. The complementary use of visual and chemical signs to recognise conspecific males is documented for female butterflies (Silberglied and Taylor 1978; Constanzo and Monteiro 2007). Nevertheless, major differentiation of chemical profiles *per se* does not necessarily imply a more efficient mechanism of sexual isolation. Female butterflies may rely on different compound concentrations or on the presence of a few or even single constituents within the total androconial chemical blend of conspecific males for mate recognition (Hayashi et al. 1977; Yildizhan et al. 2009). Further investigation is required to elucidate the roles of individual compounds and their blends in the behaviour of the *Colias*-clade sulphurs.

The most diverse androconial blend among the species in our study belongs to *Phoebis philea*. It presents a dominance of lowly volatile compounds (those eluting later than pentacosane on a non-polar GC column), which may be a clue to behavioural aspects of courtship of this species. If in fact those compounds are involved as semiochemicals in mate recognition and quality assessment, we could expect substantial androconia-antennae contact between males and females. This behaviour was indeed reported for *P. marcellina* (Rutowsky 1983), in whose androconial secretions we identified a predominance of more volatile compounds than those of *P. philea*. Presumed lowly volatile pheromones are also found in other species of Pieridae (Rutowski 1980; Grula et al. 1980; Sappington and Taylor 1990) and Danainae (Meinwald et al. 1969; Meinwald et al. 1974; Schulz et al. 1993), in which direct contact occurs during courtship (Pliske and Eisner 1969). On the other hand, high volatile compounds were characteristic of particular species such as limonene in *P. marcellina* and farnesene in *A. menippe* and *P. marcellina*. It is believed that butterflies also use volatiles as mating cues at close-range communication (Boppré 1984). Therefore, such volatiles may not be negligible just because of their low concentrations in relation to the major compounds.

Few of the major compounds found in our study were previously reported in butterflies: 13-methylpentacosane as a contact sex pheromone in the sulphur *Colias philodice* (Grula et al. 1980), hexadecanoic acid in the male hairpencils of many species of Danaini (Schulz et al. 1988; Schulz et al. 1993) and 2-phenylethyl hexadecanoate as a major androconial compound in the nymphalid genus *Bicyclus* (Wang et al. 2004). On the other hand, many compounds we identified in sulphurs were previously unknown from Pieridae and Lepidoptera. For minor blend constituents, this may be due to advances achieved in the analytical methodology when compared to the results of previous exploratory works on the androconial chemistry of Pieridae, which date to ca. 40 years ago (Hayashi et al. 1977; Kuwahara 1979; Grula et al. 1980; Honda

and Kawatoko 1982). Nonetheless, in the case of major constituents, because we are dealing with species of a poorly investigated Neotropical clade from a chemistry standpoint, a number of novelties were arguably expected to occur. For example, as far as we are aware, benzyl salicylate has not been recorded as an lepidopteran semiochemical to date. It is nonetheless reported as a male sex pheromone component of the Florida woods cockroach (*Eurycotis floridana* Walker, 1868; Farine et al. 1994) and a constituent in floral scents across different families of angiosperms (Knudsen et al. 2006). It is present in three of the six *Colias*-clade species in our investigation, being a major constituent in the androconial secretions of *A. menippe* and dominant in *P. marcellina*. The compound has fixative properties (Sturm and Peters 2007), which could arguably interfere with the volatility and tenacity of other compounds in the androconial blends.

When compared to recent phylogenies of the group (Wahlberg et al. 2014; Murillo-Ramos et al. 2018; Núñez et al. 2020), the chemical similarity of the androconial blends reflected the phylogenetic proximity between the two investigated species of *Anteos.* However, neither the phylogenetic relationships among the four species of *Phoebis* or between the two genera were supported. When treated as a whole, a moderate correlation between genetic distance and chemical dissimilarity was in fact noticed for the group, but aside the co-occurrence of a dominant unidentified aliphatic ester between the species of *Anteos*, assumed to be an informative synapomorphy, no such feature occurred among *Phoebis*. Because we are dealing with elements directly involved in mating and species recognition, a strong differentiation in such features is expected among closely related species in order to ensure reproductive isolation. Therefore, our results did not yield evidence of a conclusive phylogenetic signal on the chemical composition of androconial secretions for the *Colias*-clade butterflies.

Conclusions and perspectives

The investigation on the scale morphology of males evidenced androconial patches that are clearly differentiated from the non-androconial wing surface and exhibit particular features that could facilitate the release of semiochemicals, such as higher density of scales, higher length and larger windows in the upper lamellae, in comparison to the ordinary scales. Furthermore, the analyses of the androconial extracts of all six studied species revealed a wide array of species- and gender-exclusive compounds – some of which novel for Lepidoptera. The species-specific nature of the blends of the sympatric and ecologically similar species point towards the value of such diversification in mating-oriented strategies of butterflies. Nevertheless, a number

of questions arose from our results: are all the androconial chemical components involved in sexual interactions or are some rather cues used in male-male communication? Are they directed exclusively to mate recognition or are there compounds that indicate male fitness/quality? Do they act as a backup for visual stimuli or do they provide distinct information? And, on which sensory cues, visual or chemical, do female butterflies rely more strongly? Some of these questions were assessed for Neartic pierids, and conclusions varied among species. Very diverse and species-specific androconial chemical blends occur in the *Colias*-clade sulphurs of northeastern Brazil; we expect that a wide array of different strategies are involved in their sex-oriented chemical communication as well.

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Author Contributions

Carlos Eduardo B. Nobre conceived, designed and performed the research, collected and analyzed the data, contributed with reagents and materials, prepared figures and tables, authored the drafts of the paper, approved the final draft.

Layse A. S. Lucas contributed with analysis tools, analyzed the data, reviewed the drafts of the paper, approved the final draft.

Rafael J. R. Padilha contributed with materials and analysis, prepared figures and approved the final draft.

Daniela M. A. F. Navarro contributed with reagents, materials and analysis tools, and approved the final draft.

Luiz C. Alves contributed with reagents, materials and equipment, and approved the final draft.

Artur C. D. Maia conceived and designed the research, analyzed the data, contributed with reagents and materials, edited figures, reviewed the drafts of the paper, approved the final draft.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: BOLD Systems number of the specimens used for the Mantel analysis.

Species	BOLD specimen number
Phoebis philea	ASARD2112-12
Phoebis philea	ASARD2111-12
Phoebis philea	ASARD2113-12
Phoebis philea	ASARD2114-12
Phoebis philea	ASARD2115-12
Phoebis philea	MHMXM532-07
Phoebis philea	USLEP1567-101
Phoebis philea	LNAUU1421-15
Phoebis philea	GBGL6644-09
Phoebis philea	BLPDO834-10
Phoebis philea	ASARD2839-12
Phoebis philea	LNAUU1422-15
Aphrissa statira	BCIBT2009-19
Aphrissa statira	ASARD2925-12
Aphrissa statira	ASARD2127-12
Aphrissa statira	ASARD2126-12
Aphrissa statira	ASARD2125-12
Aphrissa statira	ASARD2124-12
Aphrissa statira	ASARD2123-12
Aphrissa statira	ASARD2122-12
Aphrissa statira	ASARD2121-12
Phoebis argante	LEPAR492-11
Phoebis argante	BICBT1933-19
Phoebis argante	BICBT1933-19
Phoebis argante	BICBT174-09
Phoebis argante	BICBT166-09
Phoebis argante	BICBT160-09
Anteos clorinde	MHAPA081-05
Anteos clorinde	TBGS007-09
Anteos clorinde	LYPIE151-09
Anteos clorinde	ASARD2102-12
Anteos clorinde	TBGS008-09
Anteos clorinde	LYPIE150-09
Anteos clorinde	LYPIE149-09
Anteos clorinde	LYPIE147-09
Anteos clorinde	GBGL26273-19
Phoebis marcellina	BBLWU269-09
Phoebis marcellina	BBLOD1876-11
Phoebis marcellina	BBLOC600-11
Phoebis marcellina	BBLOC574-11
Phoebis marcellina	AWCL023-09
Phoebis marcellina	ASARD2922-12
Phoebis marcellina	ASARD2138-12
Phoebis marcellina	ASARD2118-12
Phoebis marcellina	ASARD2117-12
Phoebis marcellina	ASARD2116-12

SUPPLEMENTARY MATERIAL

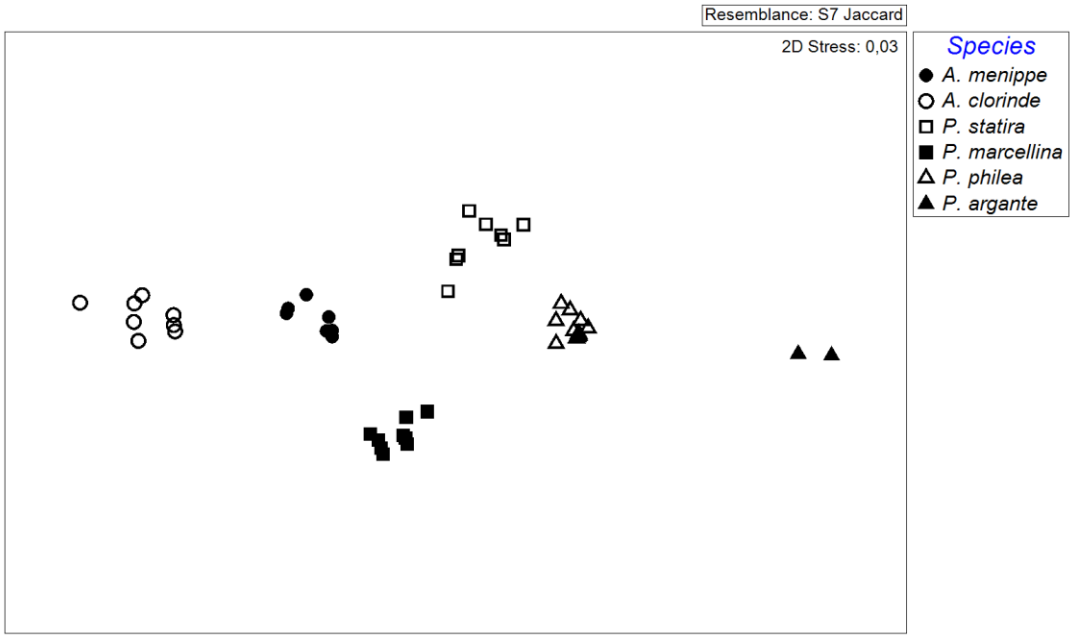
Supplementary Table 2. Total relative amounts (%) of representative compounds isolated from androconial extracts of males of the Colias-clade butterflies of northeastern Brazil. Values between brackets indicate the number of analysed samples in which the compound was present, per investigated species. * indicates that the compound identity was verified by Dr. Stefan Schulz.

Kovats Index	Compound	*Anteos menippe* (n=10)	*Anteos clorinde* (n=8)	*Phoebis statira* (n=9)	*Phoebis marcellina* (n=10)	*Phoebis philea* (n=12)	*Phoebis argante* (n=5)
Terpenoids							
Monoterpenes							
1032	D-Limonene	-	-	-	0.90 [5]	-	-
Sesquiterpenes							
1514	(*E*,*E*)-α-Farnesene	0.70 [6]	-	-	1.51 [8]	-	-
Diterpenes							
2038	(*E*,*E*)-Geranyl linalool	-	-	-	3.81 [10]	-	-
Aliphatic compounds							
Branched alkanes							
2532	13-Methylpentacosane *	-	28.30 [8]	-	-	-	-
Poliunsaturated hydrocarbons							
2837	Squalene	-	-	-	2.56 [4]	-	-
Ketones							
1847	Phytone	-	-	-	-	-	2.43 [5]
2195	Ethyl octadecanoate *	-	1.08 [5]	-	-	-	-
2381	Hexyl hexadecanoate *	-	-	-	-	-	0.59 [5]
Acids							
1760	Tetradecanoic acid *	-	-	0.81 [4]	-	-	-
1953	Hexadecenoic acid	1.61 [7]	-	0.37 [1]	49.14 [10]	0.02 [1]	-
1961	Hexadecanoic acid *	2.34 [8]	-	18.55 [8]	-	-	-
2141	Linoleic acid *	-	-	-	-	0.59 [7]	0.75 [2]
2149	Unid. aliphatic acid 1 *m/z*: 79,41,55,67,95	-	-	-	-	0.83 [8]	-
2156	Octadecenoic acid	5.18 [7]	-	-	-	0.45 [8]	-
2166	Octadecanoic acid	-	-	-	-	0.86 [8]	-
Esters							
2459	Unid. aliphatic ester 1 *m/z*: 69,55,41,68,97	1.28 [10]	-	-	-	-	-
2470	Unid. aliphatic ester 2 *m/z*: 96,81,55,97,43	20.08 [10]	-	-	-	-	-
2556	Hexyl octadecadienoate *	-	-	-	-	2.62 [12]	-
2565	Hexyl octadecatrienoate *	-	-	-	-	1.60 [12]	-
2577	Unid. aliphatic ester 3 *m/z*: 82,83,55,43,41	8.88 [10]	-	-	-	-	-
2581	Hexyl octadecanoate *	-	-	-	-	1.30 [12]	-
2582	Hexenyl octadecanoate *	-	15.33 [8]	-	-	-	-
2663	Unid. aliphatic ester 4 *m/z*: 96,55,81,97,41	4.83 [10]	0.56 [3]	-	-	-	-
2674	Unid. aliphatic ester 5 *m/z*: 96,81,97,55,43	38.15 [10]	50.72 [7]	-	-	-	-
3081	6,10,14-Trimethyl-pentadecan-2-yl tetradecanoate *	-	-	-	-	-	2.41 [5]
Alcohols							

1853	6,10,14-Trimethyl-pentadecan-2-ol *	-	-	-	-	-	88.96 [5]
Aldehydes							
1819	Hexadecanal	-	-	-	-	-	3.22 [5]
2941	Heptacosanal	1.44 [7]	-	-	-	-	-
3041	Octacosanal	0.68 [1]	2.52 [3]	-	-	-	-
Nitrogen-containing compounds							
1634	Unidentified N-cont. compound 1 * *m/z*: 146,65,120,147,92	-	-	4.51 [7]	-	-	-
1780	Unidentified N-cont. compound 2 *m/z*: 130,161,77,131,103	-	-	2.63 [5]	-	-	-
Aromatic benzenoids							
1892	Benzyl salicylate *	9.94 [10]	-	0.45 [3]	37.12 [10]	-	-
1907	Unid. aromatic benzenoid 1 *m/z*: 138,109,69,120,121	1.62 [8]	-	-	-	-	-
2585	Benzyl hexadecanoate	-	-	0.8 [3]	-	0.24 [5]	-
2686	Unid. aromatic benzenoid 2 *m/z*: 105,104,55,106,91	-	-	-	0.45 [4]	-	-
2691	2-Phenylethyl hexadecanoate *	-	-	16.66 [9]	-	-	-
2767	Benzyl linoleate	-	-	1.58 [5]	-	7.99 [12]	-
2779	Benzyl linolenate	-	-	-	-	4.76 [12]	-
2790	Unid. aromatic benzenoid 3 *m/z*: 91,55,69,97,83	-	-	-	-	3.03 [11]	-
2797	Benzyl octadecanoate	-	-	-	-	0.47 [6]	-
2823	Unid. aromatic benzenoid 4 *m/z*: 117,57,239,115,43	-	-	1.18 [3]	-	-	-
2874	Unid. aromatic benzenoid 5 *m/z*: 105,104,55,41,43	-	-	6.91 [8]	-	0.61 [12]	-
2885	Unid. aromatic benzenoid 6 *m/z*: 105,104,85,79,43	-	-	-	-	0.42 [11]	-
Unassigned compounds							
2476	Unassigned compound 1 *m/z*:85,43,101,84,55	-	-	20.83 [9]	0.17 [2]	4.78 [12]	-
2682	Unassigned compound 2 *m/z*: 85,43,101,84,57	-	-	-	0.44 [4]	4.04 [12]	-
2715	Unassigned compound 3 *m/z*: 94,79,55,95,41	3.02 [10]	-	-	-	-	-
2719	Unassigned compound 4 *m/z*: 94,79,43,95,41	-	1.5 [4]	-	-	-	-
2956	Unassigned compound 5 *m/z*: 94,43,95,134,109	-	-	21.95 [8]	-	10.3 [12]	-
2987	Unassigned compound 6 *m/z*: 91,79,67,305,108	-	-	-	-	0.29 [7]	-
3055	Unassigned compound 7 *m/z*: 117,67,115,81,95	-	-	2.76 [5]	-	-	-
3073	Unassigned compound 8 *m/z*: 94,80,43,95,55	-	-	-	-	1.04 [8]	-
3156	Unassigned compound 9 *m/z*: 94,95,135,43,67	-	-	-	-	17.46 [11]	-
3162	Unassigned compound 10 *m/z*: 43,57,55,41,105	-	-	-	3.81 [3]	-	-
3176	Unassigned compound 11 *m/z*: 94,95,43,135,109	-	-	-	-	12.31 [11]	-
3197	Unassigned compound 12 *m/z*: 94,43,134,95,109	-	-	-	-	22.92 [11]	-

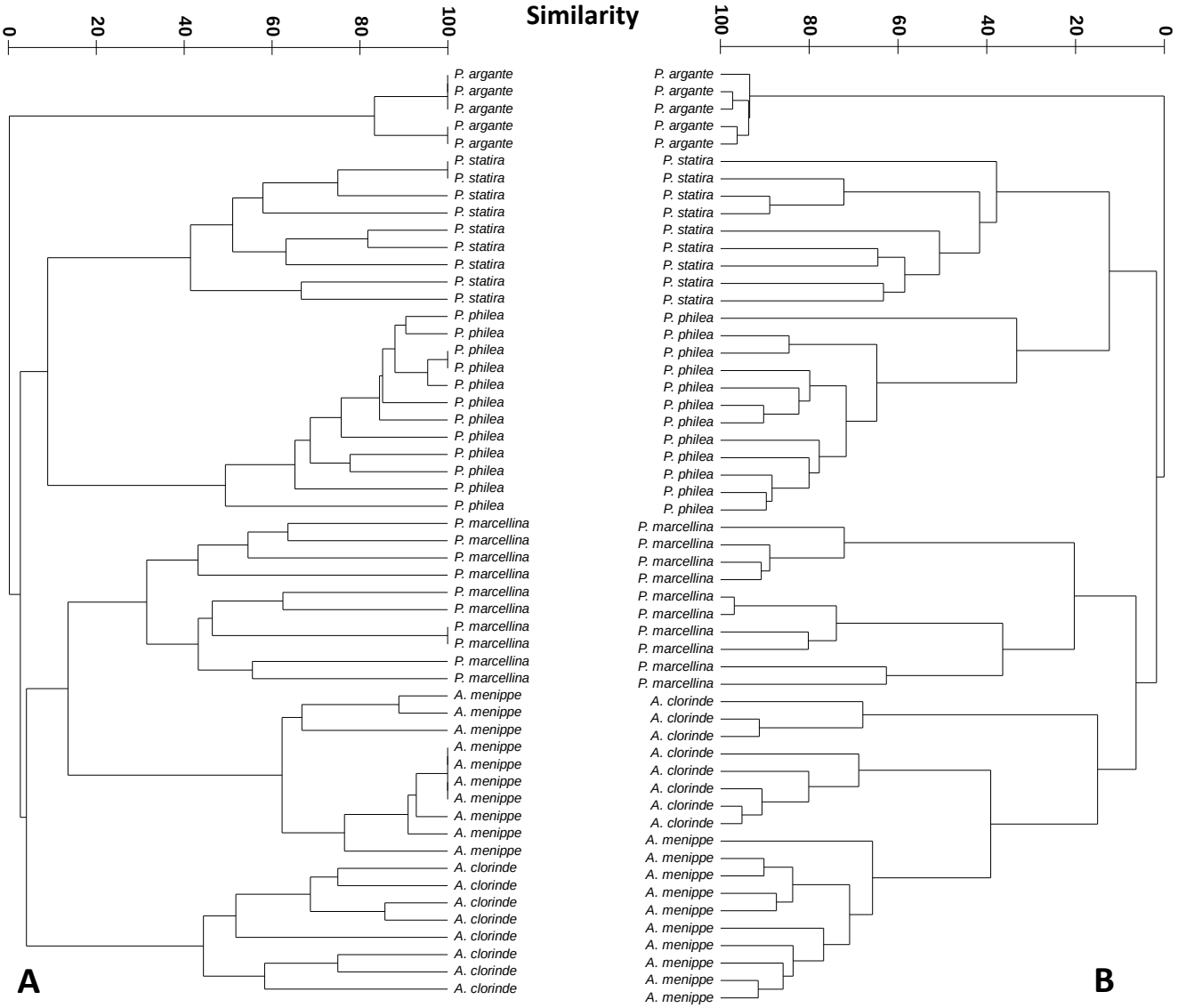
SUPPLEMENTARY MATERIAL

Supplementary Figure 1. Jaccard-based NMDS ordination for the chemical composition of androconial secretions of the Colias-clade butterflies of northeastern Brazil.



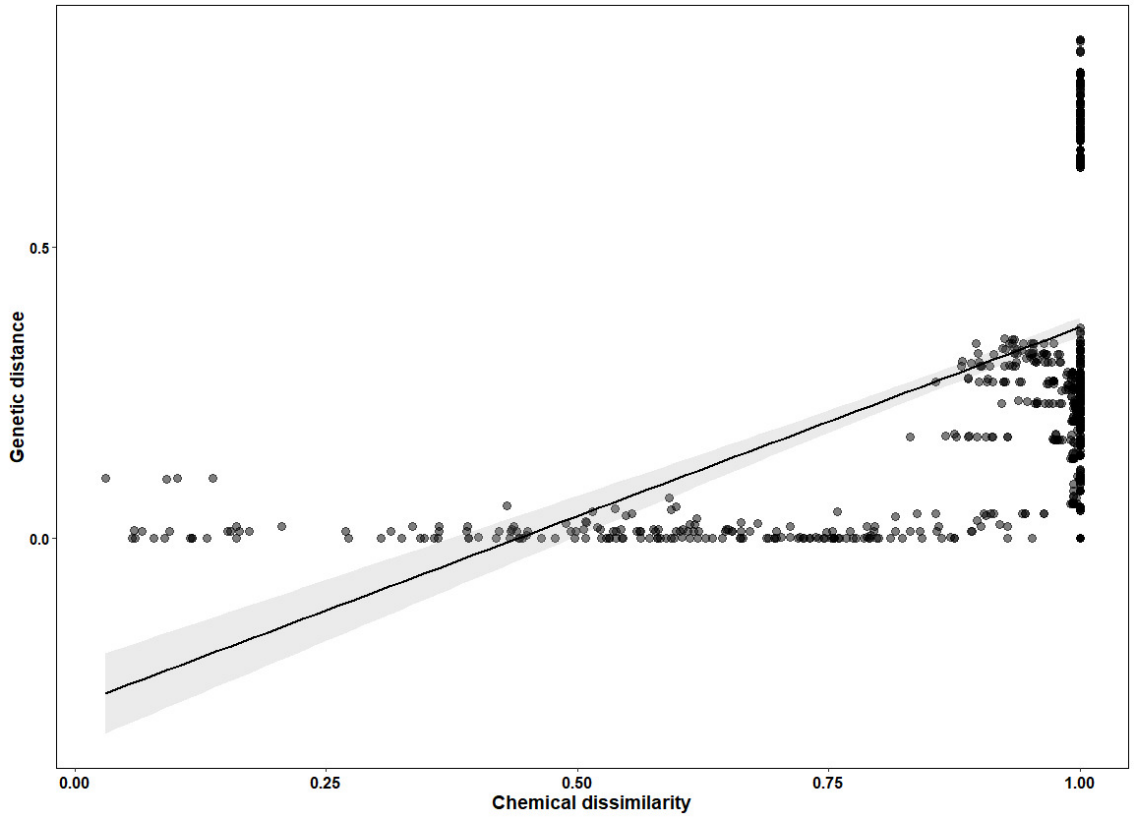
SUPPLEMENTARY MATERIAL

Supplementary Figure 2. Dendrograms based on (A) Jaccard and (B) Bray Curtis similarity indexes of the androconial chemical compounds of the *Colias*-clade butterflies of northeastern Brazil.



SUPPLEMENTARY MATERIAL

Supplementary Figure 3. Correlation between genetic distance and Jaccard-based androconial chemical dissimilarity of the *Colias*-clade butterflies of northeastern Brazil



2.2 ARTIGO 2: TAXON AND AREA-RELATED ANDROCONIAL CHEMICAL DIVERGENCES OF HELICONIINI AND ITHOMIINI BUTTERFLIES (LEPIDOPTERA: NYMPHALIDAE) IN THE ATLANTIC FOREST

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Taxon and area-related androconial chemical divergences of Heliconiini and Ithomiini butterflies (Lepidoptera: Nymphalidae) in the Atlantic forest

Short running title: Butterfly androconial chemical divergences

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Abstract

Aim: Pleistocenic refugia in the Atlantic forest hypothetically isolated butterfly populations during climatic instability periods, leading to subspecific divergence. Mimetic rings predispose difficulty in visual discernment, so chemical cues are allegedly involved in intraspecific recognition. We verified whether the androconial chemical compositions of mimetic Atlantic forest butterflies diverge according to taxon (species and subspecies) and to area-related units (populations, endemism centres, and pleistocenic refugia).

Location: Brazilian Atlantic forest.

Taxon: Heliconiini and Ithomiini

Methods: Androconial extracts of butterflies of 14 taxa (357 samples) were analysed by gas chromatography / mass spectrometry. We determined the relative percentages of each compound, from which Bray-Curtis similarity matrices and NMDS plots were performed. Differences in chemical profiles within taxon- (species and subspecies) and area-related (endemism centres, pleistocinic refugia and populations) categories were accessed through ANOSIM analyses. The geographical influence over androconial composition similarity was tested using regression analyses.

Results: A total of 112 and 58 compounds were exclusive to the androconial extracts of Ithomiini and Heliconiini, respectively. Pronounced chemical differences were observed among all species and between two subspecies of *Mechanitis lysimnia* and most androconial constituents were species-specific. Only mellein occurred in extracts of all Heliconiini species, whereas the most frequent compounds among the Ithomiini were pyrrolizidine alkaloids. Our results also revealed area-related differences among populations, endemism centers, and refugia, but not as pronounced as species-related divergences. Negative correlations between geographical distance and chemical similarity were discrete but present in all species.

Main conclusions: Species were the main predictors of androconial chemical divergence of Heliconiini and Ithomiini butterflies in our study. The high species- and sex-specific character of androconial secretions suggests their involvement in reproductive isolation. Androconial blend differences between *M. lysimnia nesaea* and *M. l. lysimnia* suggest that they may be distinct species. Atlantic forest endemism centres and areas corresponding to Pleistocenic refugia did not predict androconial divergence better than populations alone.

Keywords: Androconia, butterfly communication, centres of endemism, chemical diversity, chemical signalling, Papilionoidea, pheromones, rain forest, sex-related traits

Introduction

The ecological refugia hypothesis of the Pleistocene infers that climatic instability during this period heavily influenced the contraction and expansion of forests throughout the planet, whereas certain habitats remained more stable. These “safe” areas acted as refugia to associated biota during glaciation intervals and one major consequence was the reproductive isolation of various animal and plant populations (Moreau, 1969; Haffer, 1969; Winge, 1973; Brown, 1974; Hewitt, 2000). Even though being relatively recent events, the continued segregation would had led to the formation of races or even species that latter established a secondary contact. In tropical South America, the hypothesis was called out to explain most of the current diversity patterns observed among of birds (Haffer, 1969), reptiles (Vanzolini &

Williams, 1970), butterflies (Brown, MacDonald, & George, 1974; Brown, 1977a; Tyler, Brown & Wilson, 1994) and land plants (Prance, 1982). For the Amazon forest, however, subsequent research on the regional paleo vegetation and palynology (Mayle, Beerling, Gosling & Bush, 2004; Bush, Gosling & Colinvaux, 2007) and molecular data of mammals (Lessa, Cook & Patton, 2003) indicate that forest fluctuations were mainly restricted to ecotonal boundaries.

On the other side, recent phylogeographical and modelling studies brought out remarkable connections between predicted forest fluctuations of the Pleistocene and the species distributions/endemism patterns of the Atlantic forest (Grazziotin, Monzel, Echeverrigarauy & Bonato, 2006; Cabanne, Santos, & Miyaki, 2007; Carnaval & Moritz 2008; Carnaval, Hickerson, Haddad, Rodrigues & Moritz, 2009). This highly diverse and endemic-rich ecoregion (Myers, Mittermeier, Mittermeier et al., 2000) presents a much steeper environmental gradient than the Amazon forest, with a remarkably high latitude reach and much narrower longitudinal extension (Nimer, 1979). With a complex topography and marked seasonal regime (IBGE, 1993), it remains isolated from other humid forests of the continent by the open and semi-open Cerrado, Caatinga, and Chaco (Ab'Saber, 1977). For the Atlantic forest, the refugia hypothesis finds support and is well-illustrated by the geographic parallelism displayed by closely related species (or subspecies) across different animal and plant groups, which are restricted to certain refugia, or endemism centres (Silva & Casteleti, 2003; Silva, de Sousa, & Castelletti, 2004). Because of the high degree of human exploitation in the Atlantic forest, the formalization of the endemism centres was proposed to highlight the importance of treating such subregion differently for conservation purposes (Silva & Casteleti, 2003). The division was based on the distribution of endemic species of well-studied local groups: forest birds, primates, and butterflies. Curiously, the only butterfly database considered by Silva & Castelletti (2003) referred to the family Papilionidae, based on the work of Tyler et al. (1994). Nonetheless, an already robust and enlightening bibliography on the matter was then available, suggesting similar conclusions of regional subdivisions based on studies on Heliconiini and Ithomiini (Nymphalidae) butterflies (Brown et al., 1974; Brown, 1975; 1977a; 1980; 1981; 1982 and references therein).

The almost entirely neotropical Heliconiini and Ithomiini have been among the best studied butterfly groups in the fields of biology, ecology, systematic and coevolution, since the beginning of insect surveys in the Neotropics (Bates, 1862; Müller, 1878; Benson, 1972; Brown, 1981; Brower, Willmott, Silva-Brandão, Garzón-Orduña & Freitas, 2014; Merrill et al., 2015). Because their larvae feed on poisonous plants, adults are aposematic, and a strong

selective pressure – predation – dictates convergent evolution of warning colour patterns (Benson, 19772; Brown, 1982; DeVries, 1987). Consequently, mimetic rings develop around the most abundant or distasteful species (Brown, 1974). For many of these species, discrete but promptly recognizable colour variations are restricted to and constant within endemism centres of the Atlantic forest. These varieties are traditionally used to separate populations into subspecies and a multitude of such geographical races are taxonomically accepted (Lamas, 2004). But while allopatry itself may maintain reproductive isolation among subspecies (Garzón-Orduña & Brower, 2017), intra- and interspecific breeding still occurs among the Heliconiini and Ithomiini and can be fairly frequent in hybridization zones (Brown, 1977, Brown, 1982; Mallet, Beltrán, Neukirchen & Linares, 2007; Arias et al., 2008, Gauthier et al., 2020). Even in cases where no genetic incompatibilities arise from hybridization (McMillan, Jiggins & Mallet, 1997), resulting intermediate colour patterns are disadvantageous because of the loss of mimetic signal and consequent higher predation rates (Merrill et al., 2012). Therefore, particularly in sympatric scenarios, strong mating preference is important to avoid interbreeding (McMillan et al., 1997).

Because most butterflies are diurnal, vision plays a crucial role in their adult life, which includes mate finding and attraction (Kemp & Ruttowsky, 2011). However, among species involved in sophisticated mimetic rings, such as the Heliconiini and Ithomiini, visual recognition of mates can comprise a challenging obstacle to maintain reproductive isolation (Estrada & Jiggins, 2008; Mérot, Frérot, Lepik & Joron, 2015). As Merrill et al. (2015) pointed out for colour pattern in *Heliconius*, which is perfectly applicable to other colour-mimetic organisms: "…strong selection on a single trait (colour pattern) does not seem sufficient to complete speciation". It is when the use of associated chemical cues becomes a crucial matter in mate choice (Vane-Wright & Boppré, 1993; Mérot et al., 2015), if pre-zygotic isolation is to be achieved.

Over the past 40 years, a growing number of studies have unveiled the diversity of chemical signalling used in the communication of butterflies. Males of these diurnal lepidopterans produce semiochemicals in abdominal glands and/or in wing clusters of specialized scales termed androconia (Barth, 1960). The emission of chemicals by those structures serve three main purposes: (1) aggregation cues, which results in the formation of communal leks of various species, particularly among the Ithomiini (Haber, 1978; DeVries, 1987); (2) anti-aphrodisiacs, which are used in antagonistic behaviour between males or passed on to females during copulation to deter harassment by other males (Andersson, Borg-Karlson & Wiklund, 2000; 2003; Schulz, Estrada, Yildizhan, Boppré & Gilbert, 2008); (3) sex

pheromones, designed mainly to attract conspecific females (Grula, McChesney & Taylor, 1980; Andersson, Borg-Karlson, Vongvanich & Wiklund, 2007; Constanzo & Monteiro, 2007; Nieberding, et al., 2008; Larsdotter-Mellström et al., 2016). The production and secretion of pheromones in specialized, sex-specific structures are indicatives of their sexual purpose, especially if the chemicals are also species-specific. In fact, the sex- and species-specific nature of male androconial secretions have been repeatedly demonstrated across different butterfly families (Pliske & Eisner, 1969; Grula et al., 1980; Andersson et al., 2007; Constanzo & Monteiro, 2007, Hernández-Roldán, Bofill & Dapporto, 2014; Darragh et al., 2017; Mann et al., 2017; Nobre et al., 2021). However, this high specificity has not been studied for infraspecific taxonomical categories.

Recent studies with salamanders (Palmer, Watts, Hastings, Houck, & Arnold, 2010;), crickets (Mullen, Mendelson, Schal & Shaw, 2007), and lizards (Donihue et al., 2019) indicate that divergence in chemical signals can be a relatively fast process. Significant shifts in chemical composition may occur because the synthesis of many compounds is related to the activation/deactivation of one or few genes, so even discreet alterations in any given biosynthetic pathway may lead to the production of very distinct pheromones (Wicker-Thomas, 2011). Evidence of such recent divergence in putative male sex pheromones were documented among closely related butterflies in at least two families: Hesperiidae (Hernández-Roldán et al., 2014) and Nymphalidae (Bacquet et al., 2015).

Considering the relatively recent speciation events in the Atlantic forest and the distribution patterns of Heliconiini and Ithomiini in its different endemism centres, our objective was to investigate how the androconial chemical compositions of the butterflies diverge among taxonomic categories and among different subregions of the Atlantic forest. More specifically, we aimed to (1) identify species- and subspecies specific androconial chemical compounds of Ithomiini and Heliconiini; (2) investigate particularities in chemical composition of androconial secretions among species, subspecies, and populations of heliconiines and ithomiines for each endemism centre and refugia; and (3) analyse the relationship between geographical distance and androconial chemical divergence at the species level.

Materials and Methods

The Atlantic Forest

The highly heterogeneous forest landscape that goes through the Brazilian state of Rio Grande do Norte to Argentina and Paraguay is regarded as one of the most threatened global

hotspots of biodiversity (Myers et al., 2000). The Atlantic forest covers more than 25 degrees of latitude, from a large coastal strip to interior montane forests, which may reach continuous altitudinal gradients from sea level to 2,700 m (Rizzini, 1997; Silva & Casteleti, 2003). This biogeographical region is isolated from the other two large South American moist forests (Amazon and Andes) by a wide belt of savanna-like biomes: the Caatinga, Cerrado and Chaco, which contributes to the uniqueness of the Atlantic forest biota (Rizzini, 1997; Conservation International do Brasil et al., 2000; Myers et al., 2000).

Pleistocenic refugia and Endemism Centres

According to a robust and growing set of studies on different taxa that involves endemism rates, species distributions, geology, palynology and paleovegetation, the Atlantic forest was divided into subregions that match past climatic instability during the Pleistocene (Brown, 1975; 1977a; 2005; Brown & Ab'Saber, 1979; Prance, 1982; Soderstrom, Judziewicz, & Clark, 1988; Tyler et al., 1994; Costa, Leite, Fonseca & Fonseca, 2000; Silva et al., 2004). The hypothesized areas were validated by recent paleoclimatic models and molecular evidence (Carnaval & Moriz, 2008; Cabanne, d'Horta, Sari, Santos, Miyaki, 2008; Carnaval et al., 2009). In sum, the subregions correspond to the two most stable Pleistocenic refugia – the Pernambuco refugium in the north, and the Bahia refugium in the centre – and the third southeastern, less stable São Paulo refugium. Furthermore, Silva & Castelleti (2003) proposed a classification of Atlantic forest subregions taking into consideration not only the areas of endemism but also conservation concerns. The subregions roughly match the abovementioned refugia, with the inclusion of the Brejos Nordestinos and the Diamantina areas of endemism. The former is composed of montane moist forests and is of special interest from a speciation perspective because of its highly fragmented nature caused by the expansion of the semi-arid Caatinga during the Holocene (Oliveira, Barreto, Suguio, 1999; Silva & Castelleti, 2003). Those moist enclaves, therefore, act as contemporary refugia for the biota that depends on humid habitats.

Studied Butterflies and Sampling procedures

We sampled butterflies belonging to 10 species of Heliconiini and Ithomiini and four of these are represented by populations of two different subspecies. Sampling took place in fragments of four of the five endemism centres (from herein on, EC) of the Atlantic forest biogeographical region (Figure 1), as defined by Silva & Castelleti (2003): Bahia, Brejos Nordestinos, Pernambuco, and Serra do Mar. All these ECs harbour endemic butterflies (Brown, 1975, 1977a, 1977b, 1980; Tyler et al., 1994), some of which were recently described

or are still being so (Freitas, 2020, Freitas et al., 2020, Freitas et al., *in prep*.). We also sampled one population of *Heliconius erato phyllis* (Fabricius, 1775) in an area of the Caatinga domain that has vegetational elements of the Atlantic forest, which suggests its past relation to montane forests of the Brejos Nordestinos (Andrade, Rodal, Lucena & Gomes, 2004) (Table 1).

Table 1. Sampling localities of studied Heliconiini and Ithomiini butterflies. State acronyms: (BA) –Bahia; (PE) – Pernambuco; (SP) – São Paulo.

Endemism Centre / Refugium	**Municipality (State)**	**Sampling Area**	**Mean Temperature**	**Sampling Altitude**
Pernambuco	Recife (PE)	Dois Irmãos State Park	26 °C	0 – 90 m
Pernambuco	Igarassu (PE)	Usina São José, Piedade fragment	25 °C	45 – 150 m
Pernambuco	Jaqueira (PE)	Frei Caneca Private Reserve	22 – 24 °C	65 – 750 m
Pernambuco	São Lourenço da Mata (PE)	Tapacurá Ecological Station	23.5 °C	90 – 200 m
Brejos Nordestinos / Pernambuco	Caruaru (PE)	Serra dos Cavalos Municipal Park	24 °C	780 – 950 m
Brejos Nordestinos / Pernambuco	Taquaritinga do Norte (PE)	Mata do Flecheiro fragment	21 °C	950 – 1050 m
Bahia	Camacã (BA)	Serra Bonita Private Reserve	23.6 °C	220 – 860 m
Serra do Mar / São Paulo	Jundiaí (SP)	Serra do Japi Biological Reserve	15.7 – 19.2 °C	900 – 1200 m
Caatinga / -	Buíque (PE)	Catimbau National Park (PNC)	23 °C	800-900m

Adult specimens were collected *in situ* during flight or while visiting flowers. Contemplated taxa from Heliconiini (Heliconiinae) were: *Heliconius erato phyllis*, *He. ethilla flavomaculatus* Weymer, 1894, *He. ethilla narcaea* (Godart, 1819) and *He. sara apseudes* (Hübner, [1813]). From Ithomiini (Danainae): *Hypothyris euclea laphria* (E. Doubleday, 1847), *Hy. ninonia daeta* (Boisduval, 1836), *Hy. ninonia daetina* (Weymer, 1899), *Mechanitis lysimnia lysimnia* (Fabricius, 1793), *M. lysimnia nesaea* Hübner, [1820], *Napeogenes inachia sulphurina* H. Bates, 1862, *N. inachia grazielae* Freitas, 2020, *Ithomia agnosia agnosia* H.

Bates, 1862, *Scada reckia reckia* (Hübner, [1808]) and *S. karshina delicata* Talbot, 1932 (Figure 1).

The widespread *H. erato phyllis*, *H. sara apseudes*, *Hy. euclea laphria* and *I. a. agnosia* occur throughout the Atlantic forest, while the other species present distinct subspecies across thei Atlantic range (Lamas, 2004). Both subspecies of *M. lysimnia* co-occur in the southern portion of the Bahia EC, and therefore, one population of each was sampled in the Camacã sampling locality (Figure 1).

Although species of both tribes share similar traits such as aposematism and forest-dwelling habit, butterflies of each tribe have distinctive traits. The Ithomiini of our study are typical of shaded and cooler areas, and their hostplants are among the Solanaceae (Beccaloni, Hall, Viloria & Robinson, 2008). The androconia of ithomiines are composed of long hairlike scales concealed in membrane folds on the costal area of hindwings (DeVries, 1987). On the other hand, Heliconiini species of this study are typical of forest edges and fly under direct sunlight, and their hostplants are *Passiflora* (Passifloraceae) shrubs (DeVries, 1987; Beccaloni et al., 2008). The androconia of *Heliconius* butterflies are dorsal patches of shiny grey scales located in hindwing areas which are overlapped by forewings (Darragh et al., 2017).

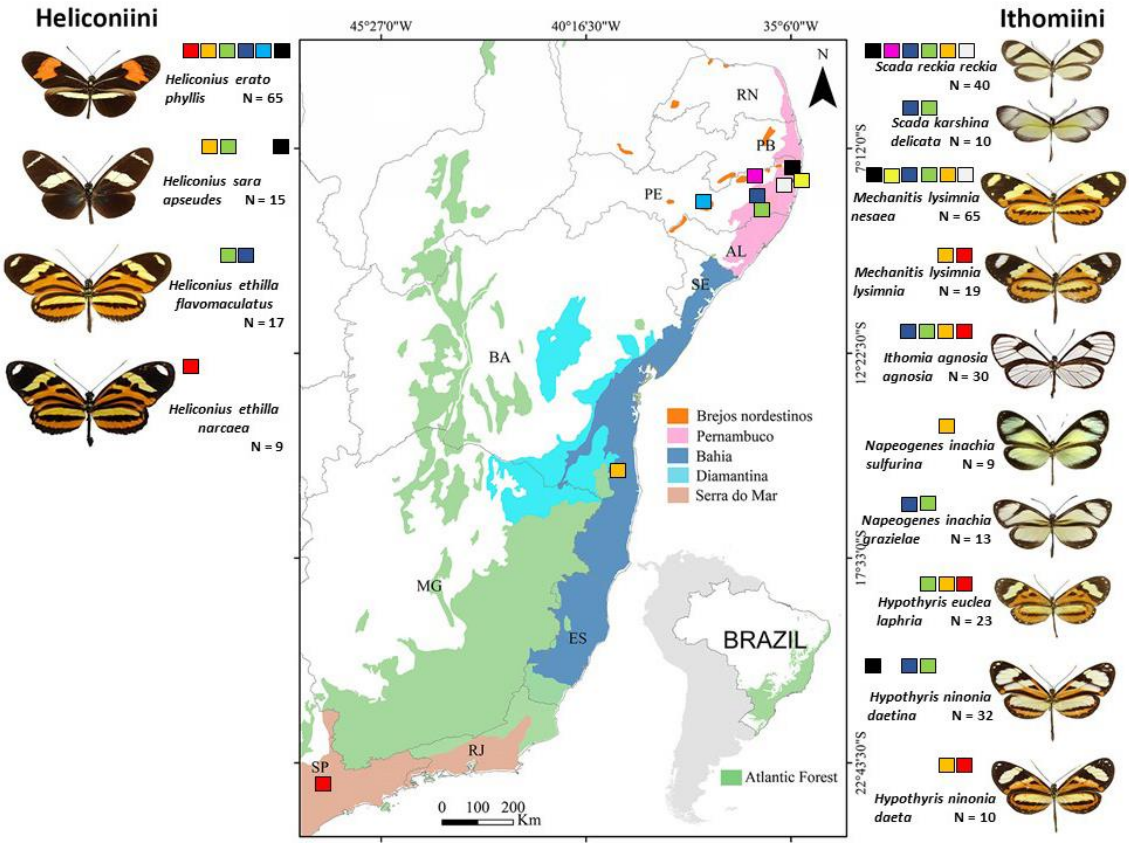

Figure 1. Map of the Atlantic forest with endemism centres and sampling localities (coloured squares) of studied Ithomiini and Heliconiini. Map adapted from Ribeiro et al. (2009).

Androconial Chemistry and Statistical Analyses

The methodology of sampling, injection, and chemical analyses of the androconial secretions followed protocols described in Nobre et al. (2021) with adaptations. To obtain samples of the androconial secretions, both wing androconia of each sampled male were excised and soaked into 1.5 mL bidistilled PA grade hexane. The extracts were filtered with silanized glass wool (Sigma-Aldrich, USA) to remove any debris, and then concentrated to approximately 25 µL under an N_2 flow. The samples were analyzed on a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent 7890A™ gas chromatograph, Agilent 5975C Series MSD™ mass spectrometer) and equipped with a non-polar HP-5ms column (Agilent J&W; 30 m × 0.25 mm i.d., 0.25 µm film thickness). A split/splitless inlet was fitted with an Agilent Thermal Separation Probe (TSP). For the analyses, 1 µL aliquots were injected into quartz micro vials which were then inserted in the TSP vial holder with the inlet set to split mode (1:1) and the GC injector temperature set to 250 °C. Exceptionally, samples of *I. a. agnosia* were injected in a 1:125 split. The GC oven temperature started at 60 °C for 2 min and then increased at a rate of 10 °C.min^{-1} to 280 °C. The final temperature was held steady for 12 min. Helium (He) carrier gas flow was maintained at a constant pressure of 7.3 psi. MS source and quadrupole temperatures were set at 230°C and 150°C, respectively. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1.0 scan.s^{-1} from *m/z* 35–450. To exclude non-androconial compounds from our analyses, the same extraction procedure was adopted to excised wing areas of conspecific females (n = 2 / population) and non-androconial wing areas of males (n = 2 / population). Solvent negative controls were also taken for each sampling event (n = 14).

Following the obtention of chromatograms, the peak areas were integrated with the MSD ChemStation E.02.01.1177 software (Agilent Technologies, Palo Alto, USA) to obtain the total ion current signals. A series of linear alkanes (C7 – C40) was used to determine the retention indices (RIs) of each compound (Van den Dool & Kratz, 1963), which were then tentatively identified by comparing their mass spectra and retention indices with those of reference samples available from personal and commercial mass spectral libraries (FFNSC 2, MassFinder 4, NIST14 and Wiley Registry™ 9th ed.). The peaks exclusive to the androconial samples were used to determine the relative percentages of each compound per sample. We considered only the compounds present in more than three individuals for any taxon to avoid non-evident contaminants. Samples from immature or old individuals, clearly identified by the

absence of typical compounds that are present in sexually mature individuals of the related species were excluded from the analyses.

The data were analysed from both Endemism Centre and Pleistocenic refugium points of view, by considering the Brejos Nordestinos EC firstly as a separate factor, and then jointly with the Pernambuco EC, which compose the Pernambuco refugium. The remaining sampling areas are equivalent: Camacã locality represents both Bahia EC and Refugium while Serra do Japi locality represents both Serra do Mar EC and São Paulo Refugium.

The relative amounts of androconial compounds were square-root transformed and the resulting values were used to generate a Bray-Curtis similarity matrix for each tribe. From these, NMDS plots were generated. Compounds with relative individual concentrations ≥ 1%, ≥ 5%, and ≥ 50% of total peak area were classified as 'minor', 'representative' and 'major', respectively, whilst the ones with relative concentrations < 1% were referred to as 'trace' compounds. To verify differences between chemical profiles within taxonomic and area categories (i.e., subspecies, species, population, EC and refugium), ANOSIM analyses were performed considering one individual as a sampling unit. To verify possible geographical influence over androconial chemical similarity, regression analyses were performed separately for each full species with three or more sampled populations. All analyses were performed in PRIMER v6 software (Clarke & Gorley, 2006).

Results

From a total of 357 samples of the 14 analysed taxa (species and subspecies), 112 and 58 compounds were found in the androconial extracts of Ithomiini and Heliconiini, respectively (Table S1, Table S2). The number of compounds per taxon varied from an average of 37 in *Hypothyris ninonia daetina* to only three in *Heliconius sara apseudes* and *Scada reckia reckia* (Figure 2). These relatively low amounts contrast to the total number of compounds that occur in the crude chromatograms, which could have as much as 300 compounds. These last, however, include compounds which are also present in females wings and non-androconial areas of male wings. The mean number of androconial compounds was similar between subspecies of *He. ethilla* (12) and *N. inachia* (10 and 13), but divergent between subspecies of *M. lysimnia* (8 and 15) and *Hy. ninonia* (21 and 37) (Figure 2).

With average dissimilarities of more than 90%, the only shared androconial compound among Heliconiini was mellein (Table 2; Table S1). Other than that, species revealed several exclusive representative and major compounds (Figure 3).

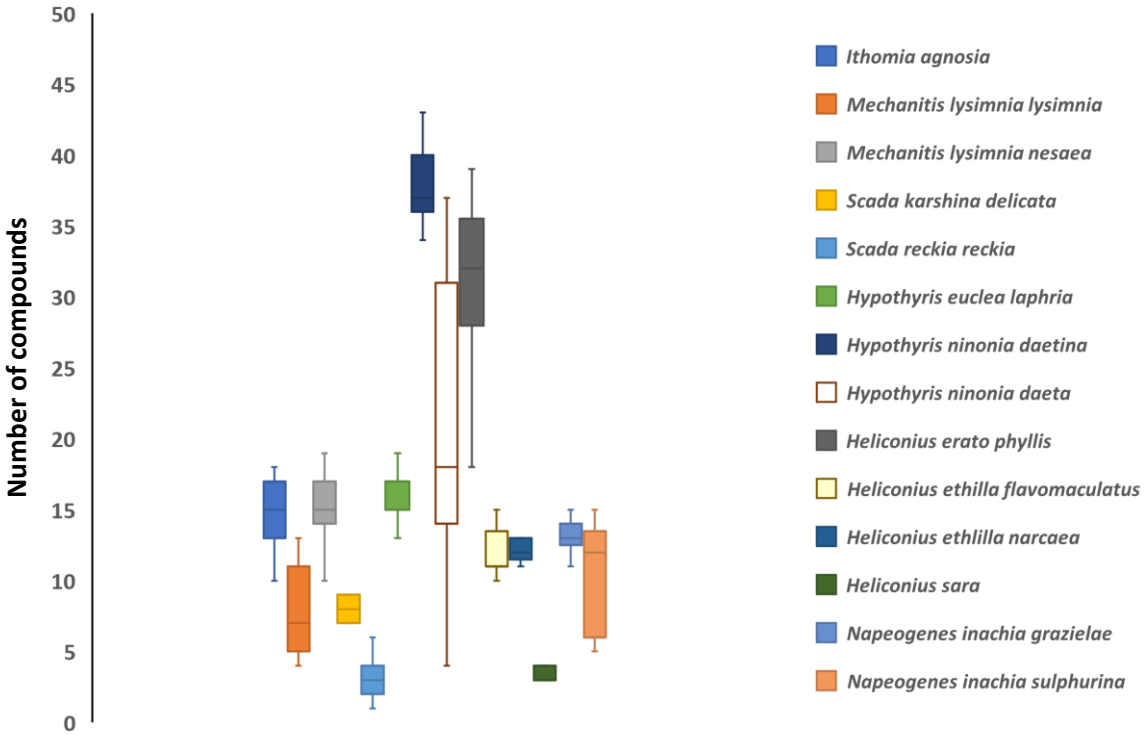

Figure 2. Number of androconial compounds by sampled taxon of Ithomiini and Heliconiini. Boxplots: median – internal line; whiskers – non-outlier range.

Among the Ithomiini, the most frequent compounds were hydroxydanaidal and an unassigned compound (Figure 4). Both were major or representative compounds across the tribe (12 taxa) but absent in the genus *Scada* (Table S2). A few important compounds were typical for genera: in *Scada* – unidentified compounds 40 and 41 – and in *Hypothyris* – β- and α-farnesene and farnesol – with relative amounts varying according to species, but the majority of androconial compounds were species-specific for the ithomiines (Table 2, Table S2, Figure 4). At the subtribe level, no compound was exclusive to the Mechanitidina and only two trace compounds were exclusive to the Napeogenina: cf. 5-cyanotropolone and cf. 4-aminopropiophenone (Table S2).

Table 2. Main androconial secretion constituents for each taxon of Heliconiini and Ithomiini. Categories after median values: * exclusive / ¤ non-exclusive to the taxon; ˣ minor; ˣ ˣ representative; ˣ ˣ ˣ major. RI: Retention index.

Taxon	Unidentified Compound	Compound or mass spectra	RI	Category
Heliconiini				
Heliconius	-	Mellein	**1565**	*, ** or **
Heliconius erato phyllis	-	Hexadecan-1-ol	**1882**	**
	28	mz 83, 55, 69, 43, 57	**2189**	**

	33	mz 85, 43, 55, 41, 69	**2659**	**
	34	mz 85, 43, 101, 84, 57	**2682**	**
	39	mz 94, 43, 95, 134, 109	**3203**	**
Heliconius sara apseudes	**-**	trans-Linalool oxide (furanoid form)	**1096**	***
	-	trans-Linalool oxide (pyranoid form)	**1179**	**
Heliconius ethilla	**-**	Heneicosene	**2078**	**
	-	Isoheptadecanol	**2144**	**
	-	Eicosen-1-ol, cis-9	**2272**	**
	-	Tricosene	**2279**	**
	-	Cf. 13-Docosen-1-ol	**2472**	**
Ithomiini				
Mechanitidina				
Scada	**40**	mz 57, 43, 85, 100, 45	**1108**	***
	41	mz 57, 43, 85, 100, 45 (Isomer II)	**1147**	***
Scada karshina delicata	**41**	mz 57, 43, 85, 100, 45 (Isomer II)	**1147**	¤¤¤
Scada reckia reckia	**-**	Methyl β-indoleacetate	**1836**	**
	76	mz 79, 67, 108, 41, 55	**2263**	**
	-	Cf. (Z)-9-Tricosene	**2271**	**
	-	Cf. Cholesta-4,6-dien-3-ol	**2912**	¤¤
	-	Cf. Cholesterilene	**2925**	**
Mechanistis lysimnia lysimnia	**-**	Cf. 9-Hexacosene	**2572**	*
	-	Cf. Heptacos-1-ene	**2675**	¤¤¤
	-	Cf. Nonacos-1-ene	**2873**	*
Mechanistis lysimnia nesaea	**55**	mz 104, 163, 132, 43, 41	**1547**	¤¤
	-	Methyl hydroxydanaidoate	**1589**	¤¤
	-	Methyl farnesoate	**1749**	¤
	-	Methyl farnesoate (isomer II)	**1792**	¤
Ithomiina				
Ithomia agnosia agnosia	**-**	Farnesyl acetate	**1842**	**
	93	mz 69, 68, 43, 67, 81	**2678**	**
	95	mz 69, 68, 43, 55,41	**2688**	**
	96	mz 68, 69, 43, 265, 41	**2703**	**
Napeogenina				
Hypothyris	**-**	Cf. (*E*)-β-Farnesene	**1462**	*
	-	Cf. (*Z*,*E*)-α-farnesene	**1498**	*
	-	Cf. (*E*,*E*)-α-farnesene	**1513**	*
	-	Cf. (2*Z*,6*E*)-Farnesol	**1729**	*
Hypothyris ninonia	**68**	mz 55, 41, 69, 83, 97	**1855**	* or **
	-	Hexadecan-1-ol	**1883**	* or **
	-	Palmitoleic acid	**1960**	¤¤
	-	Octadecenoic acid	**2040**	¤
	75	mz 43, 96, 82, 55, 81	**2209**	**
	102	mz 69, 93, 41, 81, 55	**3115**	*
Hypothyris euclea laphria	**-**	Cf. n-Hexadecyl ethanoate	**2211**	¤¤
Napeogenes inachia	**-**	Palmitoleic acid	**1960**	¤¤
	-	Hexadecanoic acid	**1966**	**
	-	Octadecenoic acid	**2040**	¤

73	mz 43, 82, 55, 96, 81	**2193**	*
-	Cf. n-Hexadecyl ethanoate	**2211**	¤

Taxon-related divergences

A definitive difference in the androconial chemistry of Heliconiini was obtained among species (ANOSIM: Global R = 1, p = 0.001) and the NMDS plot visually separated the individuals in species-related clumps (Figure 5a). The subspecies of *He. ethilla* also differed between themselves, but with a much lower R value (ANOSIM: Global R = 0.347; p = 0.001; Table S1; Figure 6a). Furthermore, the average chemical dissimilarity among species of *Heliconius* were higher than 90%, while only 15.7% of divergence was observed between *He. ethilla flavomaculatus* and *He. ethilla narcaea*.

A significant difference was also found in the androconial scent chemistry of different Ithomiini species (ANOSIM: Global R = 0.793, p = 0.001; Figure 5b), with pairwise comparisons revealing R values close to or higher than 0.9, except for the two species of *Scada* (R = 0.502, p = 0.001; Table S3). Furthermore, the overall distinctiveness among the Ithomiini was more robust when subspecies were analysed as separate entities (ANOSIM: Global R = 0.9, p = 0.001). These differences were strongly influenced by the divergence between the two subspecies of *M. lysimnia* (ANOSIM: Global R = 0.999, p = 0.001; Figure 6b), whose androconial secretions exhibited an average dissimilarity of 87.4% in their chemical profiles. In the same manner, the populations of both subspecies of *M. lysimnia* that co-occurred in Camacã were highly divergent (ANOSIM: R = 1, p = 0.001). Although males of *M. lysimnia lysimnia* and *M. lysimnia nesaea* shared the majority of androconial compounds, we found large quantitative discrepancies in concentrations of major compounds: whereas the unidentified compound 51 and the pyrrolizidine alkaloids hydroxydanaidal and methyl hydroxydanaidoate were dominant in the samples of *M. lysimnia nesaea*, they were only detected as minor or trace compounds in *M. lysimnia lysimnia* (Table S2, Figure 4). A similar divergence pattern was observed in the concentrations of both probable isomers of methyl farnesoate. On the other hand, cf. heptacosene, is dominant the samples of *M. lysimnia lysimnia*, but was almost absent in those of the other subspecies (Table 2; Table S2; Figure 4).

For the other two Ithomiini species with geographic races, only slight quantitative or qualitative divergences were found, leading to average dissimilarities of only 30% to 37% between subspecies. Likewise, comparisons between the two subspecies yielded discrete R

values (*Hy. ninonia*: R = 0.385, p = 0.002; *N. inachia*: R = 0.358, p = 0.001) and no exclusive representative or dominant compound.

Area-related divergences

With few exceptions, significant differences in androconial blend compositions were found for populations, EC's and refugia for every tested species (Table 3). However, the obtained R values were much lower than those found when species were compared. The exception here is *He. sara*, in which qualitative and quantitative differences among populations were abnormally large. This might arguably be attributed to undersampling (N = 3, 4, and 8 / population).

Furthermore, there was no indication that refugia or ECs influence androconial chemical divergence more pronouncedly than the tested populations for any given species. The exceptions were *Hy. Ninonia, He. Sara* and *M. lysimnia* for which slightly higher R values were obtained among refugium (Table 3). Pairwise analyses revealed species in which populations separated by smaller distances (< 150 km) were not chemically divergent, whereas others isolated by thousands of kilometres were (Table S4).

There were a few androconial secretion constituents exclusive to males from a single population, but these mostly appeared as trace compounds. Among representative compounds, only cf. (Z)-9-Tricosen and cf. cholesterilene were exclusive to the *S. r. reckia* population in Taquaritinga do Norte (PE), and cf. methyl 10-octadecenoate was only found in males of *S. karshina delicata* of the Jaqueira (PE) population (Table S4).

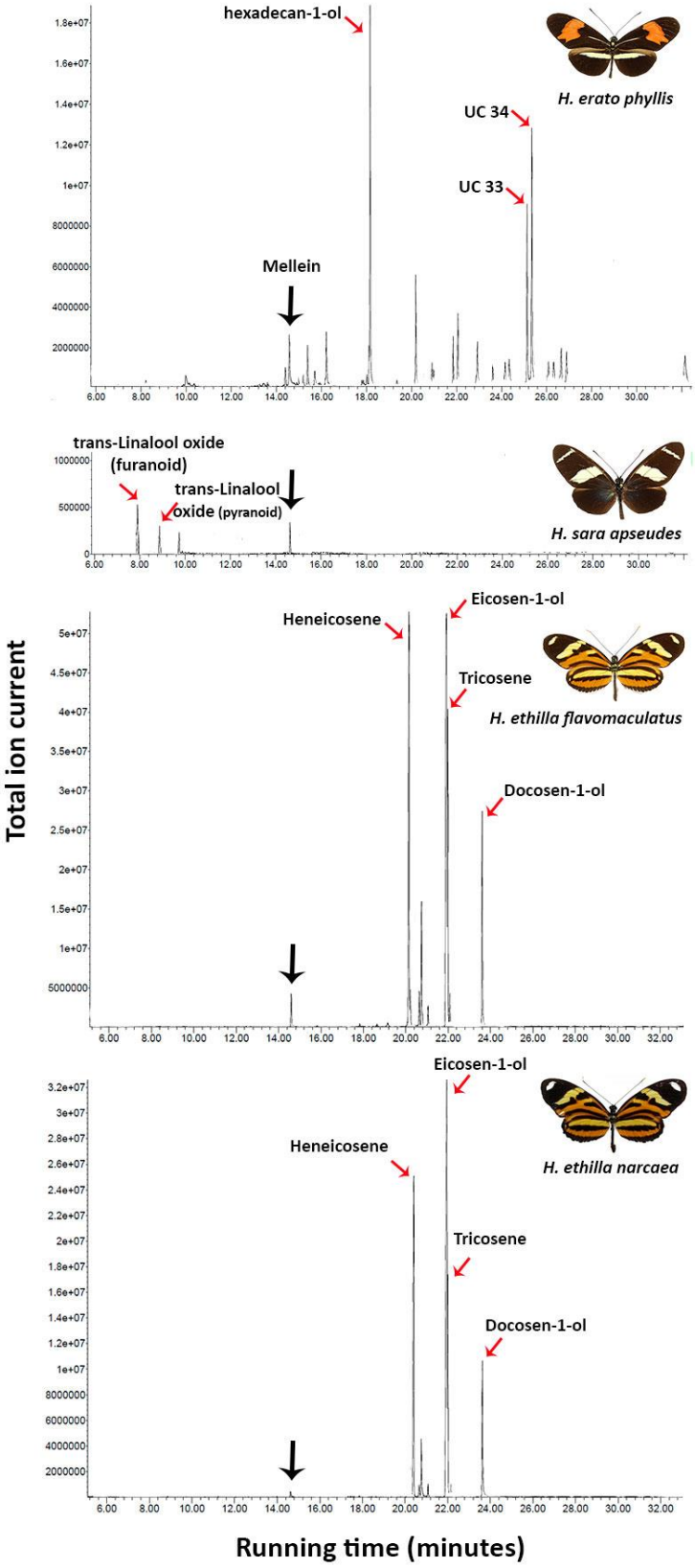

Figure 3. Illustrative chromatograms with shared (black arrows) and species-specific compounds (red arrows) for *Heliconius* species.

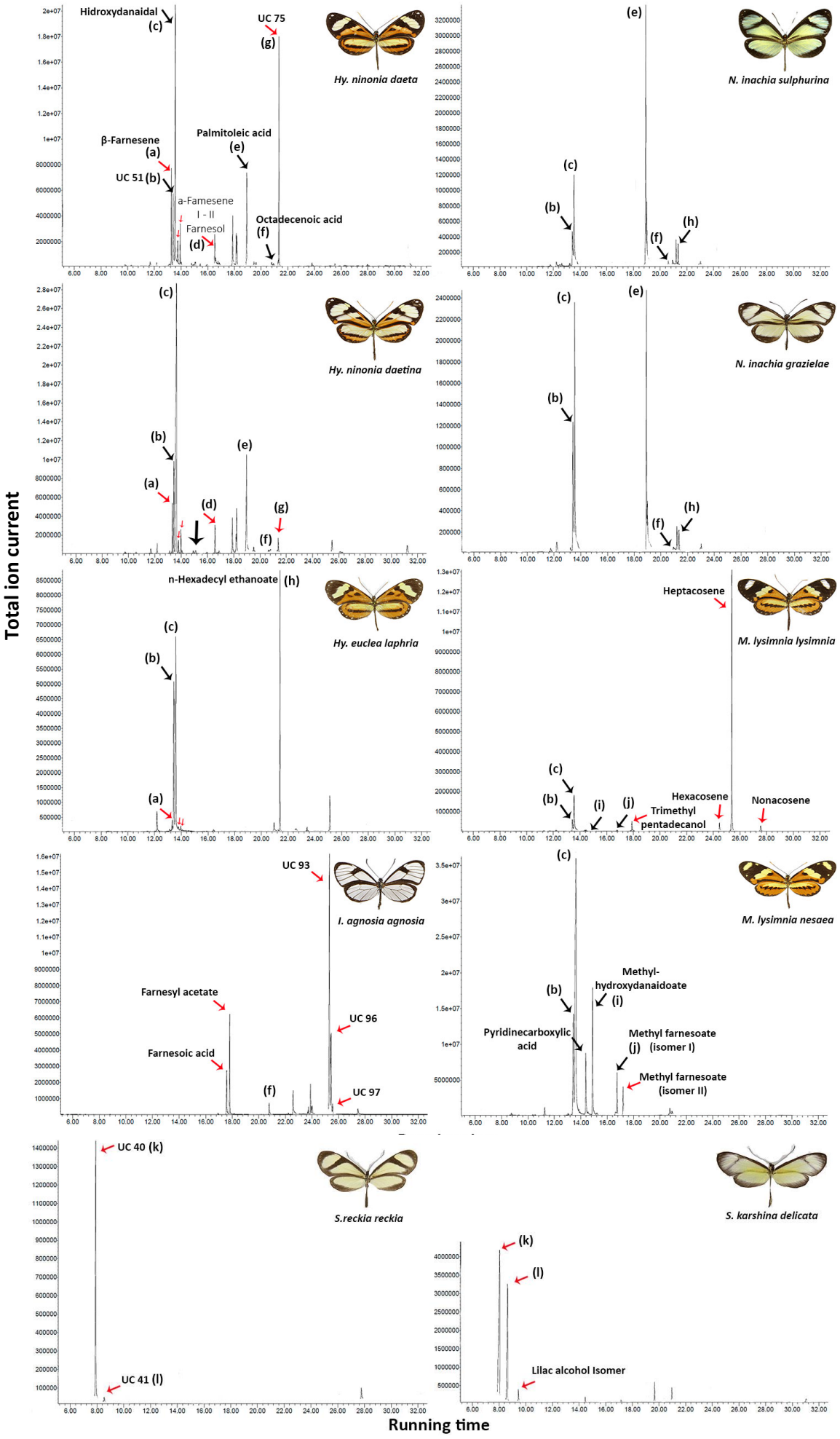
Hidroxydanaidal (c)
UC 75 (g)
β-Farnesene (a)
UC 51 (b)
Palmitoleic acid (e)
a-Famesene I - II
Farnesol (d)
Octadecenoic acid (f)
Hy. ninonia daeta
(e)
N. inachia sulphurina
(c)
(b)
(f)
(h)
(c)
Hy. ninonia daetina
(e)
N. inachia grazielae
(b)
(a)
(d)
(g)
(f)
n-Hexadecyl ethanoate (h)
Hy. euclea laphria
Heptacosene
M. lysimnia lysimnia
(i)
(j)
Hexacosene
Trimethyl pentadecanol
Nonacosene
UC 93
I. agnosia agnosia
M. lysimnia nesaea
Farnesyl acetate
UC 96
Farnesoic acid
UC 97
Methyl-hydroxydanaidoate (i)
Pyridinecarboxylic acid
Methyl farnesoate (j) (isomer I)
Methyl farnesoate (isomer II)
UC 40 (k)
S.reckia reckia
S. karshina delicata
UC 41 (l)
Lilac alcohol Isomer
Total ion current
Running time

Figure 4. Illustrative chromatograms with shared (black arrows) and main genera-, species- or subspecies-specific (red arrows) androconial compounds of the Ithomiini. UC: unidentified compound.

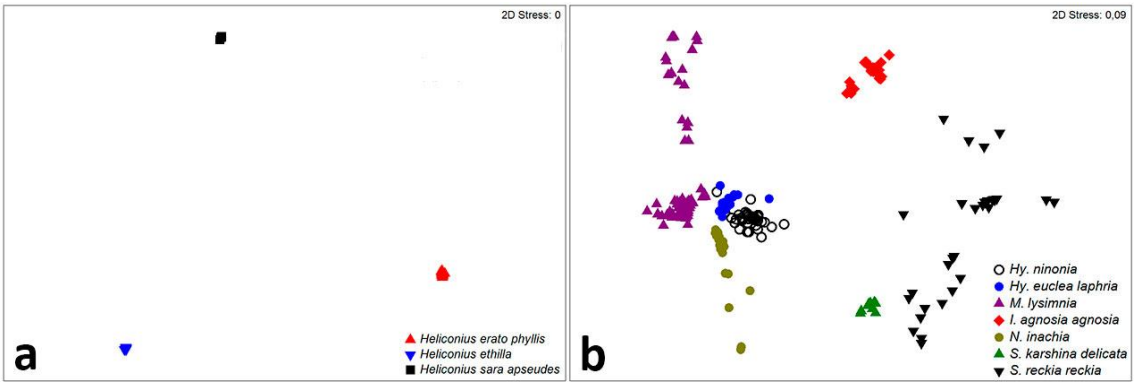

Figure 5. NMDS ordination plots for androconial chemical composition among species of (a) Heliconiini and (b) Ithomiini. Ithomiine individuals represented by triangles are Mechanitidina, by diamonds are Ithomiina and by circles, Napeogenina.

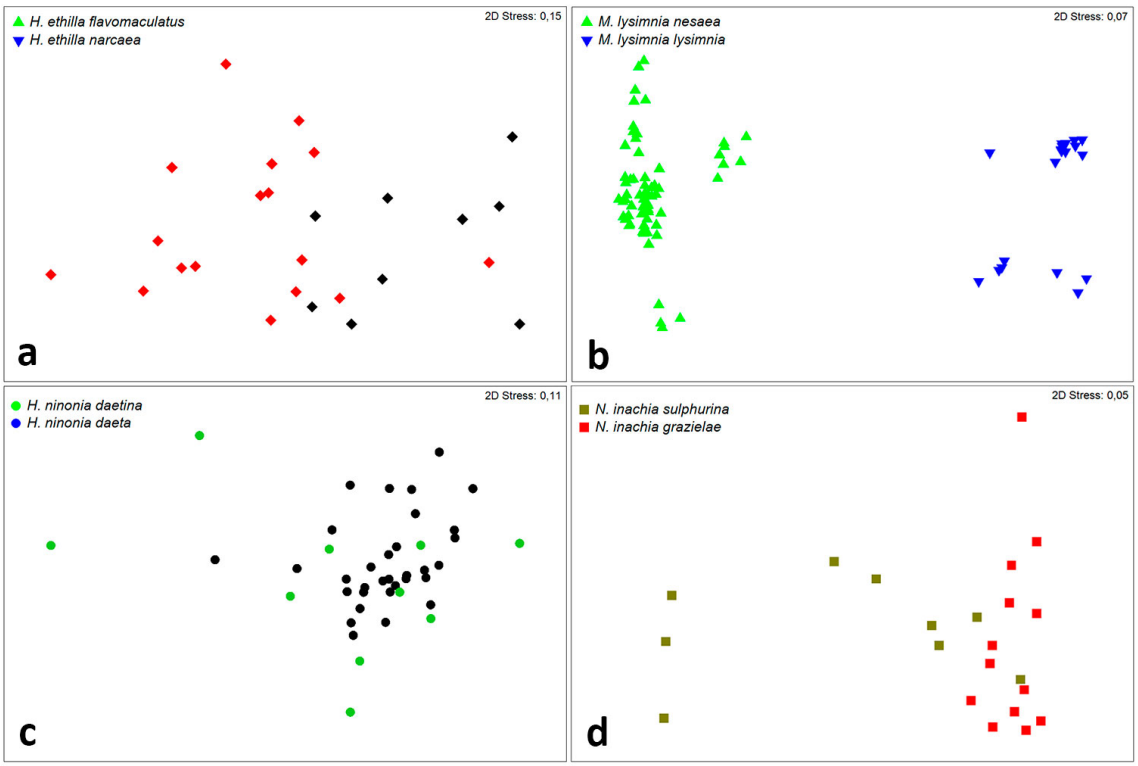

Figure 6. NMDS ordination plots for the androconial chemical compositions between subspecies of Heliconiini and Ithomiini butterflies. (a) *Heliconius ethilla*, (b) *Mechanitis lysimnia*, (c) *Hypothyris ninonia* and (d) *Napeogenes inachia.*

Table 3. ANOSIM values for androconial chemical comparisons among populations, endemism centres and refugia of Ithomiini and Heliconiini species. * One population was sampled in the Caatinga domain.

Taxon	Population		Endemism Centre		Refugium	
	R	***p***	**R**	***p***	**R**	***p***
Ithomiini						
Hypothyris euclea laphria	0.288	**0.001**	0.288	**0.001**	0.288	**0.001**
Hypothyris ninonia	0.446	**0.001**	0.315	**0.002**	0.502	**0.001**
Ithomia agnosia agnosia	0.566	**0.001**	0.566	**0.001**	0.076	0.132
Mechanitis lysimnia	0.733	**0.001**	0.698	**0.001**	0.77	**0.001**
Mechanitis lysimnia lysimnia	0.612	**0.001**	0.612	**0.001**	0.612	**0.001**
Mechanitis lysimnia nesaea	0.464	**0.001**	0.073	0.15	0.005	0.436
Napeogenes inachia	0.409	**0.001**	0.409	**0.001**	0.358	**0.001**
Scada karshina delicata	0.492	**0.008**	0.492	**0.008**	-	-
Scada reckia reckia	0.385	**0.001**	0.226	**0.002**	-	-
Heliconiini						
Heliconius erato phyllis *	0.451	**0.001**	0.253	**0.001**	0.283	**0.001**
Heliconius ethilla	0.416	**0.001**	0.416	**0.001**	0.347	**0.001**
Heliconius sara apseudes	0.815	**0.001**	0.944	**0.002**	0.944	**0.002**

Negative correlations were obtained between geographical distance and chemical similarity for all species, but R values were discrete for most of them (Figure 7). Moderate values were recovered only for *He. sara* (r = - 0.608), *He. erato phyllis* (r = - 0.409) and *M. lysimnia* (r = - 0.65). For the former, we may be considering an artifact because of the low number of analysed individuals and because samples of all three individuals from the population of Camacã (Bahia) yield mellein as the sole androconial secretion constituent. A similar correlation value was also obtained when both subspecies of *M. lysimnia* were analysed together. The composition of their androconial secretions, however, lends credence to their identification as two distinct species, which inhabits different subregions for most of their range. When only the samples of *M. lysimnia nesaea* were considered for the analyses, a much weaker correlation was obtained (r = - 0.3; Figure 7f).

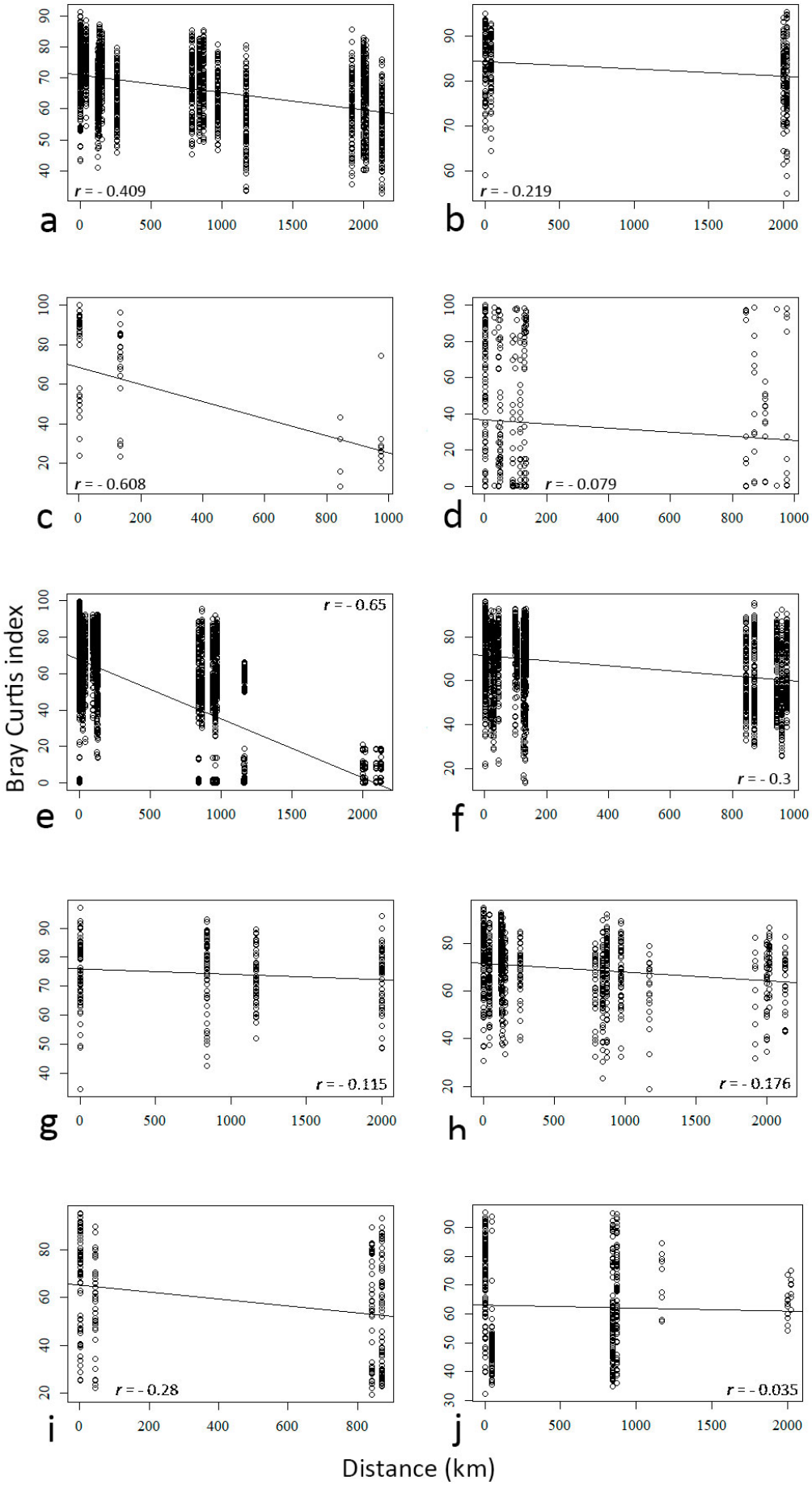
a
r = - 0.409
b
r = - 0.219
c
r = - 0.608
d
r = - 0.079
Bray Curtis index
e
r = - 0.65
f
r = - 0.3
g
r = - 0.115
h
r = - 0.176
i
r = - 0.28
j
r = - 0.035
Distance (km)

Figure 7: Scatterplots of geographic distance *vs* androconial chemical similarity, with regression values (*r*), of Heliconiini and Ithomiini butterflies in the Brazilian Atlantic forest: Heliconiini - (a) *Heliconius erato phyllis* (b) *He. ethilla* (c) *He. sara*; Ithomiini - (d) *Scada r. reckia* (e) *Mechanitis lysimnia* (f) *M. lysimnia nesaea* (g) *Hypothyris euclea laphria* (h) *Hy. ninonia* (i) *Napeogenes inachia* (j) *Ithomia a. agnosia.*

Discussion

Our study revealed widespread taxon- and biogeographic-related compositional divergences in androconial secretions among heliconiine and ithomiine butterflies in the Atlantic forest of Brazil. The degree of divergence, however, varied according to taxonomic and area-related categories.

Solid species-related differences in the composition of androconial secretions were observed in all studied butterflies. Large quantitative and qualitative interspecific differences were documented even among closely related species, as in the case of congeners in *Heliconius* and *Hypothyris*. Such clear distinctions were backed-up by R values consistently close to or greater than 0.9, and coincides with the few – but recent – results obtained in other studies that adopted similar approaches with other groups of butterflies in different biogeographic regions: Mediterranean and Continental European *Pyrgus* (Hesperiidae) (Hernández-Roldán et al., 2014), sub-Saharan *Bicyclus* (Nymphalidae) (Bacquet et al., 2015), Amazonian *Heliconius* (Nymphalidae) (Darragh et al., 2020), and *Colias*-clade butterflies in the Atlantic forest (Pieridae) (Nobre et al., 2021). The only not-so-large interspecific difference was found between two species of *Scada* (R = 0.502) and might be attributed to their similar use of resources and phylogenetic proximity (see Freitas et al., 2020). Slight indications of genetic distance as predictors of androconial secretion similarity among butterflies were found by Darragh et al. (2020) and Nobre et al. (2021). Anyway, the species-specificity of androconial blends appears to be taxonomically widespread among the Papilionoidea and there seems to be little doubt that, whenever present, androconial chemicals act as sex pheromones (Constanzo & Monteiro, 2007; Anderson et al., 2007, Nieberding et al., 2008, Darragh et al., 2017). Backed up by their high sex- and species-specific features, our results reinforce the hypothesis that such androconial secretions play a major role in the pre-zygotic isolation of butterflies.

A strongly taxon-specific outcome was also uncovered for the androconial compositions of two putative subspecies of *Mechanitis lysimnia*. Their blends significantly diverge especially in quantitative regards, as major compounds in the blend of one subspecies were identified mostly as trace constituents in the other's. A similar trend was also observed in the relative amount of compounds derived from pyrrolizidine alkaloids, which are important constituents

in the androconial secretions and chemical communication of numerous danaines and ithomiines (Brown, 1984, Hartmann, 1999, Schulz et al., 2004). Although these compounds appeared as major constituents in the androconial extracts of *M. l. nesaea*, only traces of these metabolites were detected in samples of *M. l. lysimnia*. Accordingly, hydroxydanaidal was identified by Schulz et al. (2004) as a trace compound in the androconial blends of a Brazilian population of *M. lysimnia*. Such high divergences in androconial chemistry point towards complete reproductive isolation between the two investigated subspecies of *Mechanitis*. Moreover, even the syntopic populations sampled in the Camacã locality – which in theory would have an increased chance of interbreeding – produced significantly distinct androconial blends, respective to each subspecies. Populations of *M. l. lysimnia* and *M. l. nesaea* are widely sympatric in eastern Brazil (d'Almeida, 1951). Nonetheless, Brown (1977b) stated that the occurrence of intermediate populations along this range is uncertain and suggested that behavioural or chromosomal barriers might be preventing interbreeding. In virtue of their direct association with reproductive behaviour, sex pheromones have high adaptative value and, therefore, shifts in their composition can potentially lead to speciation (Löfstedt et al., 1991). However, this is only valid if the changes in signalling are perceived by the receivers (Wyatt, 2014). To test the hypothesized reproductive isolation between the two subspecies of *M. lysimnia*, electroantennographic screenings followed by behavioural assays should be conducted. Ideally, a parallel should also be traced with molecular data. Should the species status for *M. nesaea* Hübner, [1820] is reinstated, it would be the first case in which a taxonomic recategorization would occur based on the investigation of androconial chemistry.

Differences in androconial chemistry were also observed among other pairs of subspecies sampled in our study, but to a much lower degree than those evidenced among full species or between the (now partially contested) subspecies of *M. lysimnia*. In fact, few quali-quantitative chemical divergences were evidenced between different subspecies of *Heliconius ethilla*, *Napeogenes inachia*, and *Hypothyris ninonia*. In these cases, we assume that reproductive isolation is unlikely to have been achieved because differences between subspecies were comparable to those among distinct populations of each species. We believe that although isolation periods during the Pleistocene would have influenced the current colour differentiation of populations, they would have been insufficient to produce significant shifts in metabolic routes involved in the synthesis of androconial constituents. Another possibility are post-refugial dispersion events, which would have "blurred" subspecific boundaries (Brown, 1977). Anyway, the absence of strong pheromone-mediated isolating mechanisms probably has an effect on subspecific hybridization among both heliconiines and ithomiines.

Such interbreeding is especially high in transitional zones, as described by Brown (1982) and in fact, at least for the Heliconiini, subspecies hybridization is far more common than interspecific hybridization (Mallet et al., 2007).

For the butterfly taxa analysed in our study, subregions of the Atlantic forest, either historically or ecologically defined, did not seem to predict divergences in androconial chemical composition better than populations alone. Intraspecific divergences among Refugia and ECs were similar to those between pairs of subspecies and among populations. Except for *Heliconius*, which is facing a rapid radiation process (Kozak et al., 2015), recent time-calibrated phylogenies indicate that most modern species of Nymphalidae originated in the late Miocene to early Pliocene (Wahlberg & Freitas, 2007, Elias et al., 2009, Penã, Nylin, Freitas & Wahlberg, 2010). According to the refugia hypothesis, further diversification took place latter in the Pleistocene, which would give rise to newer lineages, most of which now are considered as subspecies. As the name implies, complete speciation is thus not achieved. This is in agreement with our data, characterized by low chemical divergences between butterfly subspecies and among ECs and pleistocenic refugia, which in turn are the geographic units used to delimit subspecies.

Even though not as strong as interspecific divergences, significant interpopulation variation was evidenced in our study as well. Whatever the causes, population dissimilarities in chemical semiochemicals profiles have been demonstrated across the animal kingdom, from the sex pheromones of moths (Groot et al., 2009; Duménil et al., 2013) and lizards, (Runemark, Gabirot & Svensson, 2011, Khannoon, Lunt, Schulz & Hardege, 2013), to the cuticle hydrocarbons of crickets and stick bugs (Mullen et al., 2007; Schwander et al., 2013), and in the androconial compounds of northern Neotropical *Heliconius* butterflies (Darragh et al., 2020). Since our sampling areas have distinct biogeographic and anthropogenic histories, many factors must influence the observed chemical divergences, such as resource availability, habitat loss/fragmentation, and mimetic processes.

Most butterfly pheromones (or their precursors) are sequestered from plant chemicals through ingestion by larvae or adults (Landdolt & Phillips, 1997). As such, compound pheromone composition is directly affected by the availability of alkaloids (for pharmacophagous ithomiines and danaines) (Trigo & Brown, 1990, Honda, Honda, Yamamoto & Ômura, 2005) and hostplants (Smadja & Butlin, 2009, Darragh et al., 2019). The larvae from ithomiine and heliconiine butterfly species in our study feed exclusively in Solanaceae and Passifloraceae, respectively, but many may be polyphagous within a given genus. For example, the larvae of *M. lysimnia* and *Scada* spp. feed on a variety of *Solanum* species, according to the

area whereas those of *Heliconius* use several species of *Passiflora* as their hostplants (Beccaloni et al., 2008). Darragh et al. (2019) found that neither the major constituents nor the general composition of the androconial blend of the male *Heliconius melpomene rosina* varied according to the species of *Passiflora* in which the larvae developed; but minor constituents in the blend did. Overall, our inter-population results align with those author's findings. This opens a broad field of investigation in the Atlantic forest subregions.

Another matter beyond the direct scope of our study but likely to have influenced local chemical composition is human-driven deforestation. Habitat loss and fragmentation throughout the Atlantic forest affect butterfly communities' structure, compromising forest-dependent species (Uehara-Prado, Brown & Freitas, 2006, Filgueiras et al., 2016), reducing the genetic health of sensitive species (Massardo et al., 2000), and interfering with the diversity of mimicry rings (Uehara-Prado & Freitas, 2009). Such disruptions may be particularly critical in populations of the CEs Pernambuco and Brejos Nordestinos, where less than 16 % of the original cover remains as scattered small fragments (Ribeiro, Metzger, Martensen, Ponzoni & Hirota, 2009). Curiously, high interpopulation differences were found among spatially close (< 150 km) populations of *M. lysimnia, Hy. ninonia* and *I. agnosia*. The first two are frequently seen flying across wide open areas (C. E. B. Nobre, pers. obs.) and such small distances do not seem to impose a challenge for their dispersal (see Marini-Filho & Martins, 2010). Also, though slight, negative correlations between geographic distance and chemical similarities were obtained without exception for the studied butterflies. Whether that can be an indication of the initial stages of differentiation among populations is up to debate, but a more pronounced correlation was found in *He. erato phyllis*, whose omnipresence in the Atlantic forest range and regional fenotypic particularities may have been overlooked. Studies linking resource availability, as well as fragment distance, size, and disturbance degree to the variability in androconial composition should provide insights for the observed differences.

Because we are dealing with species involved in sophisticated mimetic rings amongst themselves and with other taxa not contemplated in our study, the very species composition of each sampling area could be influencing intraspecific androconial dissimilarities. For example, throughout the Atlantic forest, *He. erato phyllis* has an almost perfect visual co-mimic in *He. melpomene nanna* Stichel, 1899. As congeners that employ convergent visual signals, it is important that pheromonal signalling yield enough divergence to ensure reproductive isolation, since interspecific hybridization may lead to female sterility and strong selective pressures from assortative mating and predation against hybrids (for related research on *Heliconus*, see Jiggins, McMillan, Neukirchen, & Mallet, 1996, Jiggins, Naisbit, Coe & Mallet 2001). Thus, in

fragments where both species co-occur, such difference is much more imperative than where only one of them is found. Mimetic-driven influences over the androconial blends of *Heliconius* butterflies in sympatric scenarios were suggested by Mann et al. (2017) and Darragh et al. (2020), although sample sizes were too small to further substantiate the hypothesis. During our samplings and after information from published butterfly checklists (Brown, 1992, Nobre, Schlindwein & Mielke, 2008, Paluch et al. 2011, Melo, Duarte, Mielke, Robbins & Freitas, 2020), we observed that *He. melpomene nanna* did not occur in some of the study fragments or was at least much rarer than its co-mimetic *He. erato phyllis* (Jaqueira, Tapacurá, Serra do Japi, Catimbau National Park), whereas in other fragments they were equally abundant (Igarassu and Camacã). Depending on the sampled fragment, variation also occurs in the species composition of mimetic rings of *Scada r. reckia* and its co-mimics: *N. inachia sulphurina*, *N. inachia grazielae*, *S. k. karshina* (Herbst, 1792), *S. karshina delicata*, *Aeria o. olena* Weymer, 1875 and *Aeria olena* ssp. (Freitas et al., 2020); and of those species that adopt the ubiquitous tiger-like aposematic pattern, such as *He. ethilla* ssp., *Hypothyris* spp. and *Mechanistis* spp. We suggest completing the inventory of mimetic species in each of the sampling localities to further investigate influences of mimetic ring complexity over androconial chemistry in an assemblage approach.

In conclusion, we found that species were the main predictors of androconial chemical divergence of Heliconiini and Ithomiini butterflies over a considerable extension of the Brazilian Atlantic forest. Also, because of their highly species- and sex-specific nature, we endorse the hypothesis that androconial secretions contain sex pheromones involved in the reproductive isolation. By assessing the androconial blend differences of *M. lysimnia nesaea* and *M. l. lysimnia*, which are comparable to species-level divergences, we suggest they are in fact distinct species. Finally, the overall area-related differences obtained for Heliconiini and Ithomiini butterflies indicate that endemism centres and areas corresponding to Pleistocenic refugia do not predict androconial chemical divergence better than populations.

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Supporting Information

Supplementary Table S1. Median relative concentration values of androconial compounds for Heliconiini taxa in the Atlantic forest. Values between brackets indicate the number of analysed samples in which the compound was present, per investigated species.

Retention Index	Unidentified Compound number	Compound or mass spectra	*H. erato phyllis* N = 65	*H. ethilla flavomaculatus* N = 17	*H. ethilla narcaea* N = 9	*H. sara apseudes* N = 15
1096	-	trans-Linalool oxide (furanoid form)	-	-	-	52.16 [12]
1127	1	*m/z*: 43, 84, 69, 41, 57	0.86 [49]	-	-	-
1179	-	trans-Linalool oxide (pyranoid form)	-	-	-	16.20 [12]
1217	2	*m/z*: 120, 91, 119, 43, 65	-	-	-	1.63 [4]
1222	3	*m/z*: 43, 41, 57, 55, 84	0.24 [47]	-	-	-
1228	4	*m/z*: 43, 97, 41, 82, 39	2.36 [62]	-	-	-
1239	5	*m/z*: 43, 84, 71, 55, 71	0.18[15]	-	-	-
1253	6	*m/z*: 43, 84, 83, 55, 41	0.26 [20]	-	-	-
1261	7	*m/z*: 43, 95, 67, 41, 39	0.12 [32]	-	-	-
1459	8	*m/z*: 95, 43, 85, 41, 67	1.03 [62]	-	-	-
1472	9	*m/z*: 83, 43, 99, 55, 127	0.21 [39]	-	-	-
1474	10	*m/z*: 43, 104, 41, 55, 133	0.00 [8]	-	-	-
1487	11	*m/z*: 43, 125, 67, 71, 41	0.42 [53]	-	-	-
1490	12	*m/z*: 163, 43, 41, 135, 91	0.92 [8]	-	-	-
1500	-	Trans-β-Ionone	0.50 [9]	-	-	-
1551	-	Dihydroactinidiolide	1.64 [63]	-	-	-
1565	-	Mellein	6.49 [64]	2.25 [17]	1.97 [9]	32.22 [15]
1582	13	*m/z*: 43, 136, 93, 109, 121	0.38 [46]	-	-	-
1605	14	*m/z*: 43, 136, 93, 121, 109	2.33 [62]	-	-	-
1633	15	*m/z*: 43, 95, 41, 67, 82	2.25 [65]	-	-	-
1652	16	*m/z*: 69, 55, 41, 83, 67	-	0.12 [5]	-	-
1659	17	*m/z*: 161, 43, 95, 204, 105	0.91 [53]	-	-	-
1669	18	*m/z*: 43, 55, 99, 41, 81	0.72 [51]	-	-	-
1703	19	*m/z*: 43, 84, 125, 150, 107	4.51 [64]	-	-	-
1838	20	*m/z*: 95, 67, 81, 94, 68	0.36 [56]	-	-	-
1848	-	6,10,14-trimethyl-2-Pentadecanone	-	0.34 [17]	0.09 [9]	-
1853	21	*m/z*: 43, 55, 93, 41, 81	0.53 [59]	-	-	-
1869	22	*m/z*: 43, 84, 55, 85, 83	0.79 [58]	-	-	-
1882	-	Hexadecan-1-ol	9.68 [65]	-	-	-
1937	23	*m/z*: 69, 57, 41, 43, 56	-	0.13 [16]	0.27 [8]	-
1976	24	*m/z*: 55, 43, 41, 57, 83	-	0.22 [15]	0.17 [7]	-
2009	25	*m/z*: 43, 55, 41, 57, 69	1.02 [63]	-	-	-
2068	26	*m/z*: 67, 81, 82, 96, 95	-	0.15 [4]	0.20 [4]	-
2078	-	Heneicosene	-	23.43 [17]	15.76 [9]	-
2083	-	cf Octadecanol	-	2.39 [5]	1.99 [9]	-
2132	27	*m/z*: 57, 55, 43, 69, 71	-	1.78 [17]	1.71 [8]	-
2144	-	Isoheptadecanol	-	7.66. [17]	4.97 [9]	-

2177	-	Docosene	-	1.04 [11]	-	-
2189	28	*m/z*: 83, 55, 69, 43, 57	5.11 [62]	-	-	-
2086	29	*m/z*: 43, 55, 41, 81, 107	0.67 [32]	-	-	-
2194	-	Ethyl ester octadecanoic acid	0.74 [53]	-	-	-
2268	-	n-Propyl 9-octadecenoate	2.73 [61]	-	-	-
2272	-	Eicosen-1-ol, cis-9	-	37.84 [17]	48.10 [9]	-
2279	-	Tricosene	-	14.75 [17]	12.86 [9]	-
2288	30	*m/z*: 83, 55, 69, 97, 57	-	1.62 [12]	1.16 [9]	-
2291	-	Propyl ester octadecanoic acid	2.86 [64]	-	-	-
2394	31	*m/z*: 95, 43, 67, 93, 107	4.07 [64]	-	-	-
2472	-	Cf, 13-Docosen-1-ol	-	6.73 [17]	8.43 [9]	-
2477	32	*m/z*: 85, 43, 101, 84, 41	1.43 [62]	-	-	-
2543	-	1,3-Hydroxypropyl-9-octadecenoate	0.80 [27]	-	-	-
2566	-	3-hydroxypropyl-ester-octadecanoic acid	1.43 [25]	-	-	-
2659	33	*m/z*: 85, 43, 55, 41, 69	9.95 [65]	-	-	-
2682	34	*m/z*: 85, 43, 101, 84, 57	16.42 [65]	-	-	-
2756	35	*m/z*: 97, 41, 55, 43, 69	2.10 [55]	-	-	-
2777	36	*m/z*: 97, 98, 96, 43, 57	1.66 [55]	-	-	-
2808	37	*m/z*: 97, 41, 55, 98, 43	2.29 [50]	-	-	-
2829	38	*m/z*: 96, 97, 98, 43, 95	1.65 [50]	-	-	-
3203	39	*m/z*: 94, 43, 95, 134, 109	5.30 [52]	-	-	-

Supplementary Table S2. Median relative concentration values of androconial compounds for Ithomiini taxa in the Atlantic forest. Values between brackets indicate the number of analysed samples in which the compound was present, per investigated species.

Retention Index	Unidentified compound number	Compound or mass spectra	*Hypothryis*			*Mechanitis lysimnia*		*Ithomia*	*Napeogenes inachia*		*Scada*	
			Hy. ninonia daetina **N = 32**	***Hy. ninonia daeta*** **N = 10**	***H. euclea laphria*** **N = 23**	***M. l. nesaea*** **N = 65**	***M. l. lysimnia*** **N = 19**	***I. a. agnosia*** **N = 30**	***N. i. grazielae*** **N = 13**	***N. i. sulphurina*** **N = 9**	***S. karshina delicata*** **N = 10**	***S. reckia reckia*** **N = 40**
1108	40	*m/z:* 57, 43, 85, 100, 45	-	-	-	-	-	-	-	-	61.74 [10]	94.32 [14]
1147	41	*m/z:* 57, 43, 85, 100, 45 (Isomer II)	-	-	-	-	-	-	-	-	25.12 [10]	1.5 [9]
1150	-	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	-	-	-	0.18 [15]	-	-	-	-	-	-
1190	42	*m/z:* 43, 72, 57, 41, 71	-	-	-	-	-	-	-	-	0.80 [6]	-
1212	43	*m/z:* 119, 93, 43, 83, 41	0.08 [32]	0.09 [5]	-	-	-	-	-	-	-	-
1218	-	Lilac alcohol	-	-	-	-	-	-	-	-	0.09 [5]	-
1233	44	*m/z:* 43, 77, 91, 121, 93	0.06 [18]	-	-	-	-	-	-	-	-	-
1247	-	*m/z:* 41, 69, 39, 43, 55 (cf. Neral)	0.02 [20]	0.03 [4]	-	-	-	-	-	-	-	-
1252	45	*m/z:* 98, 41, 43, 69, 39	-	-	-	0.12 [12]	-	-	-	-	-	-
1264	46	*m/z:* 43, 41, 57, 55, 39	-	-	-	0.06 [8]	-	-	-	-	-	-
1275	-	*m/z:* 69, 41, 39, 84, 94 (cf. Geranial)	0.09 [31]	0.14 [4]	-	-	-	-	-	-	-	-
1318	-	4-Hydroxy-3,5,5-trimethylcyclohex-2-enone	-	-	-	0.45 [56]	0.12 [6]	-	-	-	-	-
1325	47	*m/z:* 93, 43, 121, 104, 76	-	-	0.17 [22]	-	-	-	-	-	-	-
1342	-	*m/z:* 147, 91, 63, 119, 118 (cf. 5-Cyanotropolone)	0.46 [32]	0.47 [9]	0.86 [23]	-	-	-	0.62 [13]	0.65 [6]	-	-
1349	48	*m/z:* 43, 147, 91, 41, 63	-	-	-	0.34 [60]	0.06 [10]	-	-	-	-	-
1365	-	Eugenol	-	-	-	0.11 [8]	-	-	-	-	-	-
1376	49	*m/z:* 104, 133, 105, 51, 77	1.01 [32]	0.68 [9]	1.6 [22]	-	-	-	0.67 [13]	1.07 [4]	-	-
1381	-	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	-	-	-	1.4 [62]	0.43 [5]	-	-	-	-	-
1446	-	*m/z:* 120, 65, 92, 39, 149 (cf. 4-Aminopropiophenone)	0.36 [32]	0.36 [9]	0.54 [22]	-	-	-	0.37 [13]	0.3 [5]	-	-
1452	50	*m/z:* 120, 43, 41, 39, 104	-	-	-	0.19 [58]	-	-	-	-	-	-
1462	-	(*E*)-β-Farnesene	4.64 [32]	3.79 [17]	2.23 [23]	-	-	-	-	-	-	-
1464	-	*m/z:* 155, 156, 76, 103, 102 (cf. 4,4'-Bipyridine)	-	-	0.19 [21]	-	-	-	-	-	-	-
1471	51	*m/z:* 104, 133, 105, 51, 132)	13.02 [32]	11.72 [10]	17.17 [23]	14.67 [65]	0.31 [15]	0.04 [6]	14.51 [13]	8.74 [6]	-	-

1486	-	Hydroxydanaidal	37.83 [32]	38.13 [10]	36.95 [23]	44.68 [65]	1.76 [18]	0.18 [9]	37.10 [13]	21.57 [7]	-	-
1498	-	*m/z:* 93, 119, 41, 69, 55 (cf. (*Z,E*)-α-farnesene)	1.24 [32]	1.63 [12]	1.24 [22]	-	-	-	-	-	-	-
1513	-	(*m/z:* 93, 41, 69, 107, 79 (cf. (*E,E*)-α-farnesene)	1.88 [32]	2.34 [16]	1.11 [22]	-	-	-	-	-	-	-
1518	-	*m/z:* 69, 93, 41, 79, 67 (cf. β-Bisabolene)	0.26 [28]	0.27 [8]	0.32 [15]	0.05 [28]	-	-	-	-	-	-
1520	-	*m/z:* 147, 120, 119, 64, 63 (cf. 1.3-Ethyl-1,2-benzisoxazole)	0.24 [30]	0.34 [6]	-	-	-	-	0.11 [5]	0.28 [2]	-	-
1528	-	*m/z:* 93, 41, 107, 119, 91 (cf. Bisabolene)	0.16 [6]	-	-	-	-	-	-	-	-	-
1535	52	*m/z:* 91, 69, 41, 93, 55	0.18 [7]	-	-	-	-	-	-	-	-	-
1541	53	*m/z:* 98, 43, 71, 41, 57	-	-	-	-	0.35 [11]	-	-	-	-	-
1543	54	*m/z:* 93, 107, 41, 43, 91	0.16 [7]	-	-	0.01 [9]	-	-	-	-	-	-
1547	55	*m/z:* 104, 163, 132, 43, 41	0.16 [7]	-	-	8.54 [61]	0.66 [1]	-	-	-	-	-
1550	56	*m/z:* 104, 57, 43, 133, 41	-	-	0.16 [12]	-	-	-	-	-	-	-
1551	-	*m/z:* 111, 43, 109, 137, 67 (cf. Dihydroactinidiolide)	-	-	0.09 [5]	0.67 [40]	0.15 [11]	-	-	-	0.76 [10]	-
1552	57	*m/z:* 93, 41, 91, 79, 119	0.24 [7]	-	-	-	-	-	-	-	-	-
1576	58	*m/z:* 41, 69, 43, 55, 67	0.11 [7]	-	-	-	-	-	-	-	-	-
1581	59	*m/z:* 93, 81, 41, 43, 79	0.11 [30]	0.26 [6]	-	-	-	-	-	-	-	-
1588	-	Methyl hydroxydanaidoate	0.14 [28]	0.29 [4]	-	11.81 [61]	0.27 [1]	-	-	-	-	-
1599	-	Methyl hydroxydanaidoate Isomer II	-	-	-	0.77 [13]	-	-	-	-	-	-
1602	60	*m/z:* 107, 135, 41, 93, 91	0.39 [32]	0.33 [6]	-	-	-	-	-	-	-	-
1619	61	*m/z:* 86, 43, 71, 41, 84	-	-	-	0.27 [16]	-	-	-	-	-	-
1635	62	*m/z:* 93, 41, 43, 55, 79	0.10 [30]	0.14 [4]	-	-	-	-	-	-	-	-
1676	63	*m/z:* 120, 41, 43, 55, 119	0.18 [32]	0.33 [6]	-	-	-	-	-	-	-	-
1729	-	*m/z:* 69, 41, 93, 81, 121 (cf. (2*Z*,6*E*)-Farnesol)	1.43 [28]	3.03 [16]	-	-	-	-	-	2.56 [2]	-	-
1730	-	*m/z:* 149, 121, 93, 120, 65 (cf. 2,3-Dihydroindole-4-ol-2-one)	3.22 [31]	2.30 [8]	2.87 [21]	2.46 [57]	-	-	1.13 [5]	-	-	-
1749	-	*m/z:* 69, 41, 121, 81, 114 (cf. Methyl farnesoate)	-	-	-	3.09 [61]	0.23 [9]	-	-	-	-	-
1751	-	*m/z:* 69, 41, 84, 81, 55 (cf. (E,E)-Farnesal)	0.23 [32]	0.61 [15]	-	-	-	-	-	-	-	-
1758	-	Tetradecanoic acid	0.32 [23]	1.05 [16]	-	-	-	-	0.6 [5]	-	-	-
1761	64	*m/z:* 69, 41, 109, 195, 123	-	-	-	-	-	0.62 [5]	-	-	-	-
1790	65	*m/z:* 55, 41, 69, 74, 43	-	-	-	-	-	-	-	-	0.65 [10]	-
1792	-	*m/z:* 69, 114, 41, 81, 121 (cf.Methyl farnesoate,isomer II)	-	-	-	2.97 [60]	-	-	-	-	-	-
1804	66	*m/z:* 124, 43, 41, 55, 166	-	-	-	0.41 [18]	-	-	-	-	-	-

1820	-	Farnesoic acid	-	-	-	-	-	0.57 [18]	-	-	-	-
1836	-	Methyl β-indoleacetate	-	-	-	-	-	-	-	-	-	40.8 [35]
1842	-	Farnesyl acetate	-	-	-	-	-	9.2 [8]	-	-	-	-
1843	67	*m/z:* 148, 120, 93, 179, 121	-	-	-	0.77 [39]	-	-	-	-	-	-
1852	-	*m/z:* 57, 45, 55, 43, 71 (cf. 6,10,14-Trimethyl-pentadecan-2-ol)	-	-	-	2.95 [1]	0.76 [15]	-	-	-	-	-
1855	68	*m/z:* 55, 41, 69, 83, 97	3.39 [31]	7.16 [15]	-	-	-	-	-	-	-	-
1883	-	Hexadecan-1-ol	1.70 [32]	6.87 [15]	-	-	-	-	-	-	-	-
1960	-	Hexadecenoic acid (Palmitoleic acid)	11.10 [31]	13.04 [15]	-	-	-	-	33.37 [13]	29.74 [9]	-	-
1966	-	Hexadecanoic acid	-	-	-	-	-	-	5.54 [11]	18.61 [9]	-	-
2009	69	*m/z:* 43, 55, 69, 41, 83	0.51 [27]	0.67 [14]	-	-	-	0.05 [11]	-	-	-	-
2034	70	*m/z:* 43, 95, 55, 41, 67	-	-	-	-	-	-	-	-	0.29 [10]	-
2036	-	Cf. Heptadecenoic acid	0.27 [22]	0.23 [4]	-	-	-	-	-	-	-	-
2040	-	Octadecenoic acid	0.68 [23]	1.07 [14]	-	-	-	-	-	-	-	-
2103	-	*m/z:* 55, 69, 41, 43, 74 (cf. Methyl 10-octadecenoate)	-	-	-	-	-	-	-	-	-	-
2133	71	*m/z:* 55, 57, 41, 69, 83	0.60 [19]	4.76 [12]	-	-	-	-	0.53 [6]	-	-	-
2141	72	*m/z:* 55, 41, 69, 43, 83	1.40 [29]	-	-	-	-	-	0.84 [4]	1.92 [9]	-	-
2145	-	9,12,15-Octadecatrienoic acid	-	1.36 [5]	-	2.69 [41]	-	1.35 [26]	-	-	-	-
2160	-	Octadecanoic acid	-	-	0.94 [18]	1.66 [48]	0.08 [3]	-	0.5 [8]	2.14 [9]	1.70 [9]	-
2193	73	*m/z:* 43, 82, 55, 96, 81	-	-	-	-	-	-	3.22 [13]	4.30 [8]	-	-
2202	74	*m/z:* 43, 55, 82, 41, 96	0.28 [32]	0.54 [12]	-	-	-	-	-	-	-	-
2209	75	*m/z:* 43, 96, 82, 55, 81	2.61 [31]	6.15 [16]	-	-	-	-	-	-	-	-
2211	-	*m/z:* 43, 83, 97, 69, 55 (cf n-Hexadecyl ethanoate)	-	-	27.62 [23]	-	-	-	1.68 [13]	4.46 [8]	-	-
2236	76	*m/z:* 69, 41, 55, 68, 67	-	-	-	-	-	0.12 [4]	-	-	-	-
2254	77	*m/z:* 69, 55, 41, 68, 67	-	-	-	-	-	0.06 [5]	-	-	-	-
2261	78	*m/z:* 68, 69, 41, 43, 57	-	-	-	-	-	0.07 [5]	-	-	-	-
2263	79	*m/z:* 79, 67, 108, 41, 55	-	-	-	-	-	-	-	-	-	23.9 [8]
2271	-	*m/z:* 55, 97, 83, 57, 43 (cf. (Z)-9-Tricosene)	-	-	-	-	-	-	-	-	-	18.4 [8]
2302	80	*m/z:* 69, 81, 43, 41, 93	-	-	-	-	-	0.23 [5]	-	-	-	-
2347	-	*m/z:* 55, 69, 41, 43, 83 (Eicosenoic acid)	-	-	0.74 [12]	-	-	2.8 [23]	-	-	-	-
2395	81	*m/z:* 55, 43, 82, 69, 41	-	-	-	-	-	-	0.42 [11]	0.53 [6]	-	-
2411	82	*m/z:* 43, 55, 83, 69, 41	-	-	-	-	-	-	0.22 [7]	0.53 [4]	-	-
2448	83	*m/z:* 69, 55, 41, 83, 68	-	-	0.37 [8]	-	-	0.61 [20]	-	-	-	-
2463	84	*m/z:* 68, 69, 41, 55, 43	-	-	-	-	-	0.17 [11]	-	-	-	-
2476	85	*m/z:* 69, 43, 41, 55, 67	-	-	-	-	-	0.22 [19]	-	-	-	-

2488	86	*m/z:* 68, 69, 43, 41, 55	-	-	-	-	-	0.88 [30]	-	-	-	-
2499	87	*m/z:* 83, 55, 41, 43, 69	0.33 [28]	0.48 [4]	-	-	-	-	-	-	-	-
2507	88	*m/z:* 69, 41, 55, 68, 67	-	-	-	-	-	1.53 [30]	-	-	-	-
2517	89	*m/z:* 69, 55, 41, 68, 67	-	-	-	-	-	0.47 [27]	-	-	-	-
2523	90	*m/z:* 68, 69, 55, 41, 43	-	-	-	-	-	1.34 [29]	-	-	-	2.61 [5]
2572	-	*m/z:* 97, 57, 83, 55, 69 (cf 9-Hexacosene)	-	-	-	4.26 [1]	1.44 [6]	-	-	-	-	-
2580	91	*m/z:* 105, 55, 104, 43, 41	0.27 [23]	-	-	-	-	-	-	-	-	-
2650	-	*m/z:* 69, 43, 57, 55, 41 (cf. Heptacosanal)	-	-	0.64 [17]	-	-	-	-	-	-	-
2653	92	*m/z:* 69, 68, 41, 55, 67	-	-	-	-	-	3.33 [13]	-	-	-	-
2675	-	*m/z:* 97, 83, 57, 43, 55 (cf. Heptacos-1-ene)	-	-	-	0.27 [2]	96.27 [19]	-	-	-	-	-
2678	93	*m/z:* 69, 68, 43, 67, 81	-	-	-	-	-	41.76 [30]	-	-	-	-
2687	94	*m/z:* 104, 105, 69, 68, 43	0.35 [31]	0.19 [4]	-	-	-	-	-	-	-	-
2688	95	*m/z:* 69, 68, 43, 55,41	-	-	-	-	-	9.85 [30]	-	-	-	5.57 [7]
2703	96	*m/z:* 68, 69, 43, 265, 41	-	-	-	-	-	21.41 [30]	-	-	-	-
2719	97	*m/z:* 69, 68, 55, 41, 43	-	-	-	-	-	0.97 [23]	-	-	-	-
2739	98	*m/z:* 135, 69, 134, 41, 55	0.12 [24]	0.11 [3]	-	-	-	-	-	-	-	-
2769	99	*m/z:* 169, 43, 95, 55, 57	-	-	0.45 [8]	-	-	-	-	-	-	-
2772	-	Decanoic acid, hexadecyl ester	0.14 [24]	0.15 [5]	-	-	-	-	-	-	-	-
2871	100	*m/z:* 69, 43, 68, 41, 55	-	-	-	-	-	2.7 [29]	-	-	-	-
2873	-	*m/z:* 57, 83, 97, 55, 69 (cf. Nonacos-1-ene)	-	-	-	-	1.08 [19]	-	-	-	-	-
2912	-	*m/z:* 135, 366, 143, 43, 57 (Cholesta-4,6-dien-3-ol, (3β)	0.22 [19]	0.74 [9]	-	-	-	-	-	-	-	11.15 [20]
2925	-	*m/z:* 368, 147, 43, 81, 105 (cf. Cholesterilene)	-	-	-	-	-	-	-	-	-	6.74 [12]
3007	101	*m/z:* 69, 41, 55, 93, 81	0.2 [18]	0.17 [5]	-	-	-	-	-	-	-	-
3106	-	mw 215, 43, 55, 57, 81 (cf. Cholestan-3-ol)	-	-	-	-	-	-	-	-	0.62 [6]	-
3115	102	*m/z:* 69, 93, 41, 81, 55	2.37 [32]	3.5 [11]	-	-	-	-	-	-	-	-

Supplementary Table S3. Pairwise ANOSIM values and significance levels (*p*) for the androconial chemical compositions of Heliconiini and Ithomiini species of the Atlantic forest.

Groups	**R**	***p* (%)**
Heliconiini		
H. erato phyllis, H. ethilla flavomaculatus	1	0.1
H. erato phyllis, H. ethilla narcaea	1	0.1
H. erato phyllis, H. sara apseudes	1	0.1
H. ethilla flavomaculatus, H. ethilla narcaea	0.347	0.2
H. ethilla flavomaculatus, H. sara apseudes	1	0.1
H. ethilla narcaea, H. sara apseudes	1	0.1
Ithomiini		
Hy. ninonia daetina, Hy. ninonia daeta	0.385	0.1
Hy. ninonia daetina, Hy. euclea laphria	0.993	0.1
Hy. ninonia daetina, M. lysimnia nesaea	0.993	0.1
Hy. ninonia daetina, M. lysimnia lysimnia	1	0.1
Hy. ninonia daetina, I. agnosia agnosia	1	0.1
Hy. ninonia daetina, N. inachia grazielae	0.993	0.1
Hy. ninonia daetina, N. inachia sulphurina	0.993	0.1
Hy. ninonia daetina, S. karshina delicata	1	0.1
Hy. ninonia daetina, S. reckia reckia	0.91	0.1
Hy. ninonia daeta, Hy. euclea laphria	0.952	0.1
Hy. ninonia daeta, M. lysimnia nesaea	0.986	0.1
Hy. ninonia daeta, M. lysimnia lysimnia	1	0.1
Hy. ninonia daeta, I. agnosia agnosia	1	0.1
Hy. ninonia daeta, N. inachia grazielae	0.875	0.1
Hy. ninonia daeta, N. inachia sulphurina	0.916	0.1
Hy. ninonia daeta, S. karshina delicata	1	0.1
Hy. ninonia daeta, S. reckia reckia	0.858	0.1
Hy. euclea laphria, M. lysimnia nesaea	0.981	0.1
Hy. euclea laphria, M. lysimnia lysimnia	1	0.1
Hy. euclea laphria, I. agnosia agnosia	1	0.1
Hy. euclea laphria, N. inachia grazielae	0.973	0.1
Hy. euclea laphria, N. inachia sulphurina	0.98	0.1
Hy. euclea laphria, S. karshina delicata	1	0.1
Hy. euclea laphria, S. reckia reckia	0.915	0.1
M. lysimnia nesaea, M. lysimnia lysimnia	0.999	0.1
M. lysimnia nesaea, I. agnosia agnosia	1	0.1
M. lysimnia nesaea, N. inachia grazielae	0.983	0.1
M. lysimnia nesaea, N. inachia sulphurina	0.994	0.1
M. lysimnia nesaea, S. karshina delicata	1	0.1
M. lysimnia nesaea, S. reckia reckia	0.969	0.1
M. lysimnia lysimnia, I. agnosia agnosia	1	0.1
M. lysimnia lysimnia, N. inachia grazielae	1	0.1
M. lysimnia lysimnia, N. inachia sulphurina	1	0.1
M. lysimnia lysimnia, S. karshina delicata	1	0.1
M. lysimnia lysimnia, S. reckia reckia	0.907	0.1
I. agnosia agnosia, N. inachia grazielae	1	0.1

I. agnosia agnosia, N. inachia sulphurina	1	0.1
I. agnosia agnosia, S. karshina delicata	1	0.1
I. agnosia agnosia, S. reckia reckia	0.873	0.1
N. inachia grazielae, N. inachia sulphurina	0.358	0.1
N. inachia grazielae, S. karshina delicata	1	0.1
N. inachia grazielae, S. reckia reckia	0.897	0.1
N. inachia sulphurina, S. karshina delicata	1	0.1
N. inachia sulphurina, S. reckia reckia	0.892	0.1
S. karshina delicata, S. reckia reckia	0.502	0.1

Supplementary Table S4. Pairwise ANOSIM values and significance levels (*p*) for the androconial chemical compositions of Heliconiini and Ithomiini populations of the Atlantic forest.

Taxon	Population	R	*p* (%)	Endemism Center	R	*p* (%)	Refuge	R	*p* (%)
Heliconius erato	Jaqueira-PE, PNC-PE	0.26	0.1	Pernambuco, Semiarid	0.15	5.5	Pernambuco, Semiarid	0.08	20.3
	Jaqueira-PE, Igarassu-PE	0.69	0.1	Pernambuco, Serra do Mar	0.44	0.1	Pernambuco, São Paulo	0.48	0.1
	Jaqueira-PE, Japi-SP	0.51	0.1	Pernambuco, Brejos Nordestinos	0.03	27.9	Pernambuco, Bahia	0.16	7.3
	Jaqueira-PE, Caruaru-PE	0.19	0.2	Pernambuco, Bahia	0.14	6	Semiarid, São Paulo	0.56	0.1
	Jaqueira-PE, Camacã-BA	0.18	0.2	Semiarid, Serra do Mar	0.56	0.1	Semiarid, Bahia	0.32	0.1
	PNC-PE, Igarassu-PE	0.74	0.1	Semiarid, Brejos Nordestinos	0.25	0.4	São Paulo, Bahia	0.44	0.1
	PNC-PE, Japi-SP	0.56	0.1	Semiarid, Bahia	0.32	0.1			
	PNC-PE, Caruaru-PE	0.25	0.2	Serra do Mar, Brejos Nordestinos	0.58	0.1			
	PNC-PE, Camacã-BA	0.32	0.1	Serra do Mar, Bahia	0.44	0.1			
	Igarassu-PE, Japi-SP	0.67	0.1	Brejos Nordestinos, Bahia	0.28	0.2			
	Igarassu-PE, Caruaru-PE	0.75	0.1						
	Igarassu-PE, Camacã-BA	0.53	0.1						
	Japi-SP, Caruaru-PE	0.58	0.1						
	Japi-SP, Camacã-BA	0.44	0.1						
	Caruaru-PE, Camacã-BA	0.28	0.1						
Heliconius ethila	Igarassu-PE, Caruaru-PE	0.40	0.1	Pernambuco, Brejos Nordestinos	0.40	0.1	Pernambuco, São Paulo	0.347	0.1
	Igarassu-PE, Japi-SP	0.47	0.1	Pernambuco, Serra do Mar	0.47	0.1			
	Caruaru, Japi-SP	0.41	0.1	Brejos Nordestinos, Serra do Mar	0.41	0.1			
Heliconius sara	Jaqueira-PE, Igarassu-PE	0.59	1	Pernambuco, Bahia	0.944	0.2	Pernambuco, Bahia	0.944	0.2
	Jaqueira-PE, Camacã-BA	1.00	2.9						
	Igarassu-PE, Camacã-BA	1.00	0.6						
Mechanistis lysminia	Recife-PE, Jaqueira-PE	0.52	0.1	Pernambuco, Brejos Nordestinos	0.09	16.1	Pernambuco, Bahia nesaea	0.32	0.4
	Recife-PE, Tapacurá-PE	0.72	0.1	Pernambuco, Bahia nesaea	0.35	0.2	Pernambuco, São Paulo	1.00	0.1

	Recife-PE, Igarassu-PE	0.54	0.1	Pernambuco, Serra do Mar	1.00	0.1	Pernambuco, Bahia - lysimnia	1.00	0.1
	Recife-PE, Caruaru-PE	0.64	0.1	Pernambuco, Bahia - lysimnia	1.00	0.1	Bahia nesaea, São Paulo	1.00	0.1
	Recife-PE, Camacã_nes-BA	0.72	0.1	Brejos Nordestinos, Bahia nesaea	0.42	0.1	Bahia nesaea, Bahia - lysimnia	1.00	0.1
	Recife-PE, Japi-SP	1.00	0.1	Brejos Nordestinos, Serra do Mar	1.00	0.1	São Paulo, Bahia - lysimnia	1.00	0.1
	Recife-PE, Camacã_lys-BA	1.00	0.1	Brejos Nordestinos, Bahia - lysimnia	1.00	0.1			
	Jaqueira-PE, Tapacurá-PE	0.64	0.1	Bahia nesaea, Serra do Mar	1.00	0.1			
	Jaqueira-PE, Igarassu-PE	0.24	0.3	Bahia nesaea, Bahia - lysimnia	1.00	0.1			
	Jaqueira-PE, Caruaru-PE	0.16	2	Serra do Mar, Bahia - lysimnia	1.00	0.1			
	Jaqueira-PE, Camacã_nes-BA	0.39	0.2						
	Jaqueira-PE, Japi-SP	1.00	0.1						
	Jaqueira-PE, Camacã_lys-BA	1.00	0.1						
	Tapacurá-PE, Igarassu-PE	0.69	0.1						
	Tapacurá-PE, Caruaru-PE	0.58	0.1						
	Tapacurá-PE, Camacã_nes-BA	0.70	0.1						
	Tapacurá-PE, Japi-SP	1.00	0.1						
	Tapacurá-PE, Camacã_lys-BA	1.00	0.1						
	Igarassu-PE, Caruaru-PE	0.28	0.1						
	Igarassu-PE, Camacã_nes-BA	0.36	0.1						
	Igarassu-PE, Japi-SP	1.00	0.2						
	Igarassu-PE, Camacã_lys-BA	1.00	0.1						
	Caruaru-PE, Japi-SP	1.00	0.1						
	Caruaru-PE, Camacã_lys-BA	1.00	0.1						
	Camacã_nes-BA, Japi-SP	1.00	0.1						
	Camacã_nes-BA, Camacã_lys-BA	1.00	0.1						
	Japi-SP, Camacã_lys-BA	1.00	0.1						
Hypothyris euclea	Jaqueira-PE, Japi-SP	0.43	0.6	Pernambuco, Serra do Mar	0.43	0.6			

	Jaqueira-PE, Camacã-BA	0.14	2.4	Pernambuco, Bahia	0.14	2.4			
	Japi-SP, Camacã-BA	0.38	0.2	Serra do Mar, Bahia	0.38	0.2			
Hyothyris ninonia	Jaqueira-PE, PNC-PE	0.21	10.1	Pernambuco, Semiarid	0.34	2.9	Pernambuco, Semiarid	0.51	0.4
	Jaqueira-PE, Japi-SP	0.46	3.6	Pernambuco, Serra do Mar	0.59	1.3	Pernambuco, São Paulo	0.62	0.7
	Jaqueira-PE, Igarassu-PE	0.38	0.1	Pernambuco, Brejos Nordestinos	-0.01	48.6	Pernambuco, Bahia	0.40	0.6
	Jaqueira-PE, Caruaru-PE	0.46	0.1	Pernambuco, Bahia	0.28	2.1	Semiarid, São Paulo	0.63	2.9
	Jaqueira-PE, Camacã-BA	0.26	0.5	Semiarid, Serra do Mar	0.63	2.9	Semiarid, Bahia	0.41	0.6
	PNC-PE, Japi-SP	0.63	2.9	Semiarid, Brejos Nordestinos	0.95	0.2	São Paulo, Bahia	0.37	7.5
	PNC-PE, Igarassu-PE	0.92	0.1	Semiarid, Bahia	0.41	0.6			
	PNC-PE, Caruaru-PE	0.95	0.1	Serra do Mar, Brejos Nordestinos	0.89	0.3			
	PNC-PE, Camacã-BA	0.41	0.6	Serra do Mar, Bahia	0.37	7.5			
	Japi-SP, Igarassu-PE	0.89	0.5	Brejos Nordestinos, Bahia	0.49	0.1			
	Japi-SP, Caruaru-PE	0.89	0.3						
	Japi-SP, Camacã-BA	0.37	7.5						
	Igarassu-PE, Caruaru-PE	0.25	1.2						
	Igarassu-PE, Camacã-BA	0.32	0.4						
	Caruaru-PE, Camacã-BA	0.49	0.1						
Napeogenes inachia	Jaqueira-PE, Caruaru-PE	0.80	0.1	Pernambuco, Brejos Nordestinos	0.80	0.1	Pernambuco, Bahia	0.358	0.1
	Jaqueira-PE, Camacã-BA	0.27	4.2	Pernambuco, Bahia	0.27	4.2			
	Caruaru-PE, Camacã-BA	0.37	0.2	Brejos Nordestinos, Bahia	0.37	0.2			
Ithomia agnosia	Jaqueira-PE, Caruaru-PE	0.81	0.1	Pernambuco, Brejos Nordestinos	0.81	0.1	Pernambuco, São Paulo	-0.26	100
	Jaqueira-PE, Japi-SP	0.63	16.7	Pernambuco, Serra do Mar	0.63	16.7	Pernambuco, Bahia	0.12	5.8
	Jaqueira-PE, Camacã-BA	0.63	0.1	Pernambuco, Bahia	0.63	0.1	São Paulo, Bahia	0.00	45.5
	Caruaru-PE, Japi-SP	0.00	55.6	Brejos Nordestinos, Serra do Mar	0.00	55.6			
	Caruaru-PE, Camacã-BA	0.33	0.7	Brejos Nordestinos, Bahia	0.33	0.7			

	Camacã-BA, Japi-SP	0.00	45.5	Serra do Mar, Bahia	0.00	45.5
Skada reckia	Jaqueira-PE, Caruaru-PE	0.18	7.2	Pernambuco, Brejos Nordestinos	0.24	0.1
	Jaqueira-PE, Taquaritinga-PE	0.72	0.1	Pernambuco, Bahia	0.06	25.3
	Jaqueira-PE, Camacã-BA	0.10	33.3	Brejos Nordestinos, Bahia	0.19	14.6
	Jaqueira-PE, Tapacurá-PE	-0.15	77.8			
	Jaqueira-PE, Igarassu-PE	0.30	1.9			
	Caruaru-PE, Taquaritinga-PE	0.61	0.1			
	Caruaru-PE, Camacã-BA	0.19	22.2			
	Caruaru-PE, Tapacurá-PE	0.25	25			
	Caruaru-PE, Igarassu-PE	0.19	6			
	Taquaritinga-PE, Camacã-BA	0.67	1.5			
	Taquaritinga-PE, Tapacurá-PE	1.00	9.1			
	Taquaritinga-PE, Igarassu-PE	0.51	0.1			
	Camacã-BA, Tapacurá-PE	0.00	66.7			
	Camacã-BA, Igarassu-PE	0.33	8.8			
	Tapacurá-PE, Igarassu-PE	0.43	15.4			

3 CONCLUSÕES

As conclusões que se extraem dos principais resultados de ambos os capítulos desta tese deixaram clara a natureza altamente sexo- e espécie-específica daqueles compostos que compõem as secreções androconiais das borboletas contempladas, o que sugere seu envolvimento no processo de reprodução e isolamento reprodutivo. Ademais, a condução de uma investigação morfológica mais criteriosa sobre as androcônias de pierídeos verificou que aspectos micro e macroestruturais parecem estar intimamente relacionados à estocagem e liberação dos compostos lá produzidos. Ademais, foram obtidos indicativos de que, em diferentes graus, as distâncias genética e geográfica influenciam a dissimilaridade química androconial dos pierídeos e dos heliconíneos e itomíneos, respectivamente.

Muitas das questões tratadas nessa tese eram "território desconhecido" (pelo menos no que tange aos grupos de borboletas escolhidos e à região neotropical) quando da sua elaboração, no fim do ano de 2016. Entretanto, no decorrer dos quatro anos seguintes que corresponderam ao seu desenvolvimento (2017-2020), sucedeu-se a publicação de vários trabalhos com objetivos muito similares, tendo como espécies-alvo itomíneos e heliconíneos do norte dos neotrópicos (MANN et al., 2017, DARRAGH et al., 2017, 2019, 2020; STAMM et al., 2019; MANN et al., 2020). Esses estudos, por exemplo, discutiram a microestrutura de androcônias, listaram compostos químicos androconiais, investigaram sua natureza feromonal/sexual, sua variação composicional entre populações e espécies e discutiram influências de anéis miméticos sobre essa variação. Ao mesmo tempo em que retiraram um pouco da novidade do tema, esses resultados permitiram uma discussão muito mais robusta e embasada dos nossos resultados. Também nos deixaram claro que algumas aparentes deficiências nos nossos resultados eram compartilhadas, como explicado a seguir.

Um dos objetivos iniciais desta tese era a identificação dos compostos androconiais, especialmente daqueles majoritários. Devido à novidade do tema abordado – ecologia química de borboletas neotropicais – era previsto de antemão que essa identificação seria um aspecto particularmente desafiador e exigiria parceria com especialista de formação química. No entanto, o decorrer das análises foi revelando uma crescente gama de compostos não identificados, incluindo muitos dominantes nas amostras. A consulta a especialista na área ajudou a preencher suprir diversas dúvidas, mas constatação da quantidade de compostos não identificados ao final de mais de 400 amostras de 19 espécies foi um tanto frustrante. Somam-se aí as dificuldades impostas por uma longa pandemia que vem restringindo vida e trabalho de todos os envolvidos. No entanto, a observação da grande quantidade de compostos não

identificados também nas listas de diversos *Heliconius* dos trabalhos supracitados nos deixou claro o grau de desconhecimento acerca da ecologia química das borboletas neotropicais. Essa lacuna já começou a ser preenchida, com a recente descrição de novos compostos para algumas espécies de itomíneos (STAMM et al., 2019; MANN et al., 2020; DARRAGH et al., 2021).

A correlação dos constituintes das secreções androconiais de espécies do clado *Colias* à sua distância genética sugeriu que quanto maior essa distância, maior a dissimilaridade química androconial. Essa comparação, entretanto, não levou em consideração a relação de proximidade dos compostos, o que pode ser realizada separando-os em grupos funcionais e por comprimento de cadeia, por exemplo (MANN et al., 2017). A identificação mais específica dos principais compostos encontrados possibilitará que comparações mais refinadas sejam feitas tanto para os pierídeos como para os itomíneos e heliconíneos.

Da mesma forma, experimentos de eletrofisiologia antenal e atratividade dos compostos e misturas identificados permitirá se esclarecer as funções ecológicas e comportamentais dos compostos. Dentre um rol de opções, provavelmente existirão semioquímicos de agregação (especialmente entre os Ithomiini), antiafrodisíacos, feromônios sexuais, de reconhecimento e misturas indicadoras de qualidade e fitness. A investigação tem potencial para ser levada adiante com estudos de complementariedade e preferência entre os sinais químicos e visuais. Muitas espécies ocorrem ao longo do ano e são de fácil criação em laboratório, o que otimiza a obtenção de dados. Da mesma forma, a possibilidade de experimentação de efeitos de mudança de plantas hospedeiras sobre a composição química androconial se faz oportuna, uma vez que a maioria das borboletas deste estudo têm como hospedeiras espécies de *Solanum* (Ithomiini), *Passiflora* (*Heliconius*) e *Cassia* ou *Senna* (*Anteos* e *Phoebis*) (BECCALONI et al., 2008), plantas de fácil cultivo ou coleta.

Finalmente, a comparação do grau de divergência química androconial encontrado entre espécies ao constatado para duas subespécies de *Mechanitis lysimnia* permitiu que se sugerisse que ambas podem vir a ser espécies distintas. Embora essa pareça uma exceção quando observados os resultados obtidos entre os pares das demais subespécies estudadas, a constatação abre precedentes. A presença quase universal de raças geográficas ou subespécies de borboletas ao longo da floresta atlântica e em outras unidades biogeográficas torna oportuna uma investigação mais abrangente.

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APÊNDICE A – ARTIGO PUBLICADO NO PERIÓDICO ORGANISMS DIVERSITY & EVOLUTION:

"Specialized androconial scales conceal species-specific semiochemicals of sympatric sulphur butterflies (Lepidoptera: Pieridae: Coliadinae)"

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ORIGINAL ARTICLE

Specialized androconial scales conceal species-specific semiochemicals of sympatric sulphur butterflies (Lepidoptera: Pieridae: Coliadinae)

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Abstract

Chemical cues play an important role in short-range communication of butterflies, remarkably in sexual attraction and mate choice. Differentiated scale patches on the wings of male butterflies, the androconia, are involved in the emission of pheromones. Here, we describe the androconial morphology of six sympatric species of Neotropical sulphur butterflies belonging to two genera of the *Colias*-clade (Pieridae) based on SEM imaging. Gas chromatography-mass spectrometry analyses were used to access the chemical compositions of androconial secretions, which were comparatively investigated to determine species-specific trends and to verify if they yield a phylogenetic signal. The androconial patches from all species are differentiated from the non-androconial male wing surface and exhibit morphological features that may act in both preventing the volatilization of secretions and facilitating the release of semiochemicals, such as high density and length of scales and large perforations in the upper lamellae. A total of 55 compounds were exclusive to the androconia, and unique chemical profiles are present in each butterfly species, verified through multivariate analysis. The majority of androconial compounds were autapomorphic for each species and only four were dominant in more than one species. Cluster analyses placed the two species of *Anteos* in a single clade, but otherwise evidenced low similarities in the androconial secretion compositions among species, and a moderate correlation between genetic distances and chemical dissimilarities was obtained. Our findings suggest that androconial substances are involved in mating-oriented strategies and might be associated with the evolutionary history of the reproductive isolation of sulphurs.

Keywords Butterfly communication · Papilionoidea · *Phoebis* · Phylogenetic signal · Sex pheromones · Sex-related traits

Introduction

Visual and chemical cues play complementary roles in the sexual behaviour of butterflies (Constanzo and Monteiro 2007; Smadja and Butlin 2009). While visual cues are involved in both long- and short-range intraspecific interactions, chemical cues are preponderant at close interactions among the Papilionoidea (Siberglied 1984; Vane-Wright and Boppré 1993). The relative importance of each stimulus on butterfly mate recognition and mate choice may vary according to species (Papke et al. 2007; Constanzo and Monteiro 2007), but this multimodal signalling plays a pivotal role in pre-zygotic reproductive isolation among sympatric closely related species (Bacquet et al. 2015). Thus, it is assumed that at least one of these components (e.g. colour pattern, UV reflection, pheromones) is species-specific.

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Some Coliadinae butterflies (Lepidoptera: Pieridae) are given the vernacular denomination of 'sulphurs', which is associated to the bright white/yellowish colour of their wings from the deposition of pterin pigments on the scales (Watt 1964). In northeastern Brazil, there are six sympatric species of sulphur butterflies belonging to the monophyletic *Colias*–clade (*sensu* Wahlberg et al. 2014), among which males are readily distinguished from one another by wing colour pattern. Since visual stimuli play a major role in conspecific recognition of butterflies, including Pieridae—revised on Kemp and Rutowski (2011)—, a first glance assumption can be that the pronounced differences in wing colour patterns would suffice for females to recognise conspecific males. However, males of all six species have differentiated, sex-specific scale patches on their wings—the androconia. These structures are involved in the emission of chemical compounds exclusive to male lepidopterans—especially butterflies and diurnal moths—which function as aphrodisiacs (Pliske and Eisner 1969; Nieberding et al. 2008; Yildizhan et al. 2009) or anti-aphrodisiacs (Estrada et al. 2011). Size, number and location of the androconia vary depending on the species, and their morphology appears to be related to the storage and discharge of pheromones produced by the secretory cells which are usually located at the base of the androconia (Kristensen and Simonsen 2003). For example, during courtship and for aggregation purposes, male Ithomiini butterflies (Nymphalidae, Danainae) display otherwise concealed erectile alar fringes involved in the dispersion of volatile compounds (Schulz et al. 2004). The androconia of male pierid butterflies are typically arranged in dense clusters of scales (Barth 1960) that do not have any protection to prevent evaporation of secretions besides the overlapping of the wings, when at rest. Maybe because of this lack of specialization in concealment, highly volatile androconial compounds would be disadvantageous and, in fact, the androconial chemical bouquets of pierids such as *Colias* (Coliadinae) may present large amounts of heavier and less volatile compounds (Grula et al. 1980). On the other hand, compounds with higher volatility, such as geranial and neral, occur in high concentrations in *Pieris* (Pierinae) and are released mainly during flight activity (Andersson et al. 2007).

Results are contrasting regarding a phylogenetic signal of androconial secretions in different groups of butterflies. When the chemical compounds of sympatric, mimetic species were analysed, limited phylogenetic signal was found within both Ithomiini (Schulz et al. 2004) and Heliconiini (Mann et al. 2017) (Nymphalidae: Danainae and Heliconiinae, respectively). A sophisticated convergent aposematism (e.g. bright colour, high-contrast patterns) might cause confusion in intraspecific recognition, as observed in *Heliconius* (Estrada and Jiggins 2008). Thus, it is reasonable to assume that in order to avoid hybridization, distinct chemical signatures must be adopted between closely related comimetics (Mérot et al. 2015). Nonetheless, androconial secretions of mimetic milkweed butterflies (Nymphalidae: Danainae) exhibit strong phylogenetic chemical signalling in the species level (Schulz et al. 1993). In the same way, a strong correlation was demonstrated to occur between secretions of androconia and genetic distances of partially sympatric species of *Pyrgus* (Hesperiidae: Pyrginae) (Hernández-Roldán et al. 2014). Species of this genus are morphologically very similar but do not rely on mimicry.

In this study, we present a different scenario, in which six closely related, apparently non-mimetic species belonging to the *Colias*-clade co-occur. We aimed to (1) describe the morphology of the androconia of the six species and access the biological significance of key features; (2) investigate whether there are chemical compounds exclusive to the androconial patches in relation to the remaining non-androconial wing surface and to those of the wings of conspecific females and (3) compare the composition of androconial secretions among the six species and verify whether there is phylogenetic signal for this trait.

Materials and methods

Studied butterflies

We investigated the sulphurs *Anteos clorinde* (Godart, [1824]), *A. menippe* (Hübner, [1818]), *Phoebis argante* (Fabricius, 1775), *P. philea* (Linnaeus, 1763), *P. marcellina* (Cramer, 1777) and *Phoebis statira* (Cramer, 1777) (Fig. 1). We followed the classification of Murillo-Ramos et al. (2018), who proposed *Aphrissa* as a synonym of *Phoebis*, and of Núñez et al. (2020), who raised *P. marcellina* to the species rank. All these species are typical of sunny, open areas, sympatric and to some extent, synchronic in northeastern Brazil in the same manner to what happens on most of their range (DeVries 1987; Brown Jr. 1992).

Scanning electron microscope imaging and description of androconial scales

The androconial patches and surrounding wing areas of dry mounted males belonging to the six studied species were excised from the wings using surgical scissors which were rinsed with hexane and dried between uses. The samples were placed in aluminium stubs with double-sided carbon adhesive tape and coated with 80% gold/20% palladium in a Jeol Datum Ion Sputter JFC-1100. The metalized samples were examined in a Zeiss EVO LS15 scanning electron microscope and images were obtained at an electron high tension of 10 kV. The density of scales was estimated by counting one quadrant of a 1 mm^2 area and multiplying the value by four. The length of the scales (both androconial and ordinary) was measured from base to apex for each species (mean ± standard deviation; n = 10 per species).

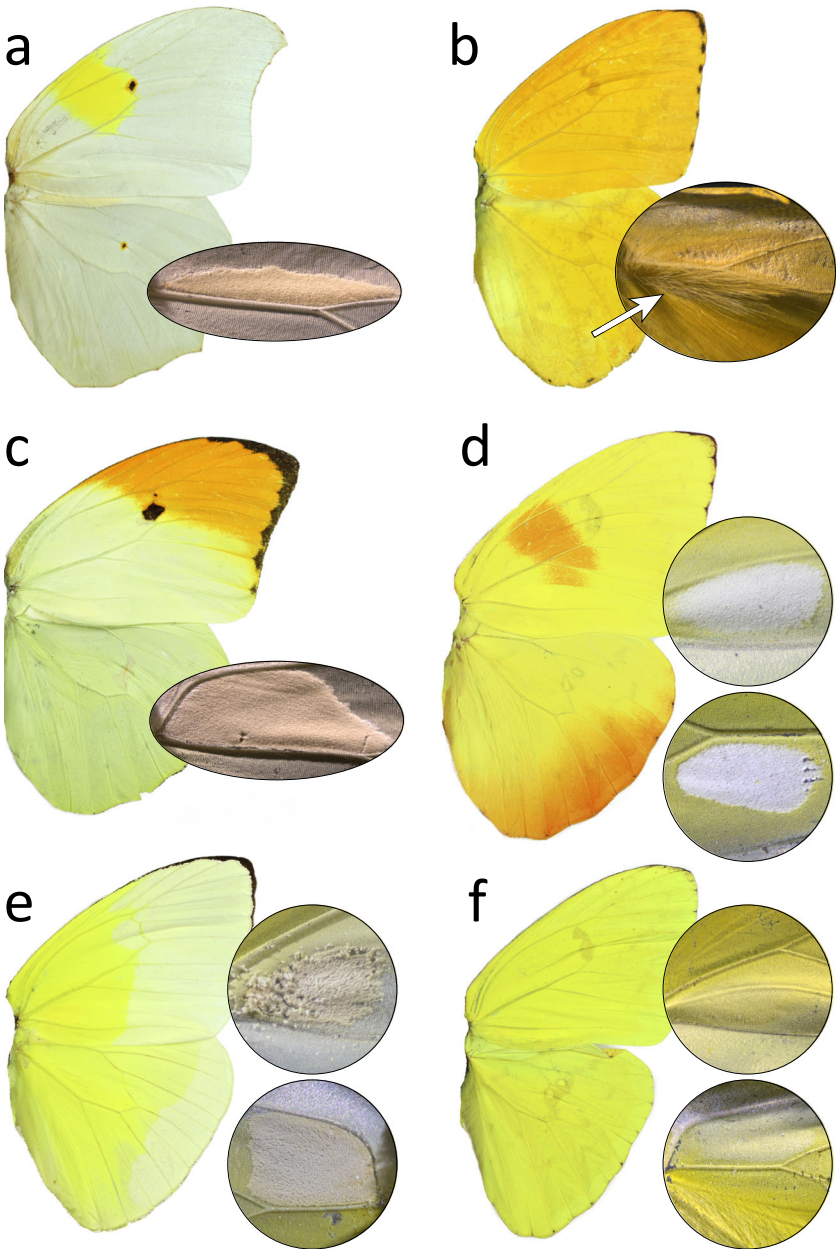

Fig. 1 Male dorsal wing colour patterns of the *Colias*-clade butterflies of northeastern Brazil: (a) *Anteos clorinde*, (b) *Phoebis argante*, (c) *A. menippe* (d) *P. philea*, (e) *P. statira,* and (f) *P. marcellina*. Details of the androconia (circles to the right), showing the shapes and colour distinction from the surrounding scales. The white arrow indicates a tuft of hair-like scales below the androconial patch

The overall shape, perforations (windows) and density of the scales surrounding the androconial patches vary noticeably, depending on the area of the wing they cover. Therefore, for comparative purposes, we considered only the ordinary scales laterally adjacent to the androconia. To describe the scales, we followed Downey and Allyn (1975) with adaptations. The nomenclature for the microstructures of the scales follows Ghiradella (1989). For the wing areas and venation nomenclature, we followed DeVries (1987).

Sampling and chemical analyses of androconial secretions

Butterflies were sampled in the municipalities of Recife (8° 03′ 10″ S, 34° 56′ 51″ W) and Buíque (8° 35′ 11″ S, 37° 08′ 52″ W), state of Pernambuco, northeastern Brazil. All individuals were captured in situ visiting flowers of *Ixora coccinea* L. (Rubiaceae) or *Bougainvillea spectabilis* Willd. (Nyctaginaceae) from 09:00 to 13:00 h.

The methodology of sampling, injection and chemical analyses of the androconial secretions was adapted from Mann et al. (2017). Immediately following capture, the individuals were sacrificed by thorax compression while inside the insect net. Androconial patches on the wings of each male (n = 5–12/species) were excised and inserted into clear 2-ml silanized glass vials containing hexane (≥ 99.7% purity, Sigma–Aldrich, USA; bidistilled prior to use). In order to latter exclude from our analyses the chemical compounds not exclusive to the androconia, the same procedure was applied to equivalent excised wing areas from conspecific

females (n = 2/species) and to non-androconial male wing areas (n = 1/species). In all cases, the butterflies were manipulated by the thorax to avoid contamination of the wings. Solvent negative controls were obtained for each sampling event (n = 6). The crude solvent extracts were filtered through a silanized glass Pasteur pipette plugged with silanized glass wool (Sigma-Aldrich, USA) to remove floating scales and other debris, then concentrated to approximately 25 μl under a laminar N_2 flow and kept at − 24 °C refrigeration until further processing procedures.

For gas chromatography-mass spectrometry (GC-MS) analyses, we used a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent 7890A™ gas chromatograph, Agilent 5975C Series MSD™ mass spectrometer) and equipped with a non-polar HP-5ms column (Agilent J&W; 30 m × 0.25 mm d.i., 0.25 μm film thickness). A split/splitless inlet was fitted with an Agilent Thermal Separation Probe (TSP). For each sample, 1 μl of the eluate was injected into quartz microvials which were then inserted in the TSP vial holder with the inlet set to split mode (1:1) and the injector temperature set to 250 °C. GC oven temperature was set at 60 °C for 2 min and then increased at a rate of 10 °C/min^{-1} to 280 °C, which was held steady for 12 min. The carrier gas flow was maintained at a constant pressure of 7.3 psi. MS Source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Mass spectra were taken at 70 eV in EI mode with a scanning speed of 1.0 scan^{-s} from m/z 35–450.

After the chromatograms were obtained, their peak areas were integrated by using the software MSD ChemStation E.02.01.1177 (Agilent Technologies, Palo Alto, USA) to obtain the total ion current signal. A homologous series of linear alkanes (C_8–C_{40}) was used to determine linear retention indices (RI) of the compounds (Van den Dool and Kratz 1963), which were then tentatively identified by comparing their mass spectra and retention indices with those of reference samples available from personal and commercial mass spectral libraries (FFNSC 2, MassFinder 4, NIST14 and Wiley Registry™ 9th ed.). Mass spectra of target compounds and their calculated retention indices were also cross referenced with data published by Hayashi et al. (1977), Grula et al. (1980), Honda and Kawatoko (1982) and Yildizhan et al. (2009). Additionally, the authors consulted mass spectrometry specialist Prof. Dr. Stefan Schulz (Technische Universität Braunschweig, Germany) for input on identifications. We only considered peaks exclusive to the androconial extracts and that were present in more than two individuals, which were used to determine the relative percentages of each compound per sample. In that way, some compounds such as the cuticular hydrocarbon 13-methylheptacosane—an important male pheromone of the sulphur *Colias philodice* Godart, 1819 (Grula et al. 1980)—were excluded from our characterization of androconial secretions because they were present in female or non-androconial male wing extracts as well. For comparative analyses of androconial extracts, we established as a threshold that compounds should be present at over 1% of the total individual peak area in at least one of the analysed samples for any species. Compounds with relative concentrations higher than 10% of total peak area were classified as 'dominant', whereas 'minor compounds' were present at concentrations between 1 and 10%. We considered compounds that eluted latter than pentacosane (RI = 2500) as lowly volatile (Table 2), according to Mann et al. (2017), which also worked on a non-polar column (HP-5ms). Non-polar GC columns are made with poorly selective stationary phases, and thus commonly applied to separate non-polar compounds roughly based on volatility.

Statistical analyses

The presence/absence of componds and their relative percentages were used to generate Jaccard- and Bray Curtis-based similarity matrices respectively, from which non-metric multidimensional scaling analyses were performed in PAST 4.02 (Hammer et al. 2001). To verify if the chemical profile within each category (i.e. species) was significantly different, ANOSIM analyses wereperformed. In order to avoid bias from excessive weight of major compounds, their relative amounts were submitted to a a priori square root transformation.

To verify whether the results reflect the phylogenetic arrangement of the studied species, resulting dendrograms were compared to the most recent phylogeny of the group (Murillo-Ramos et al. 2018) and a Mantel test was performed to correlate the matrices of chemical dissimilarity and genetic distance using the packages Vegan (Oksanen et al. 2013) and Ade4 (Dray and Dufour 2007) in R software (R Core Team 2016). The genetic distance matrix was built by calculating pairwise distances of the mitochondrial cytochrome C oxidase 1 (COI) gene using the Kimura 2-parameter (K2P) model (Kimura 1980), with the data available from the Barcode of Life Data System (BOLD) platform (Ratnasingham and Hebert 2007) (Supplementary table S1) with MEGA-X 10.1 (Kumar et al. 2018). We excluded *A. menippe* from this analysis because genetic data is not available for this species.

Results

Morphology of androconial scales

The androconia of the forewings are always on the ventral side while those of the hindwings are dorsally positioned (Table 1). Unless specified otherwise in Table 1, the scale sockets are always visible, pedicels are positioned at the basis of the median line and the longitudinal ridges on the lamina are parallel and straight (Fig. 2). The colour of androconial scales is always paler than that of the

Table 1 Morphological aspects of androconial and surrounding ordinary scales of the *Colias*-clade butterflies of northeastern Brazil

Species	Location of the Androconia	Scales											
		Androconial						Ordinary					
		Density (mm^2)	Lenght (μm)		Shape	Upper lamina		Density (mm^2)	Lenght (μm)		Shape	Upper lamina	
			Averaged	SD		Windows	Crossribs		Averaged	SD		Windows	Crossribs
Anteos clorinde†	HW: Dorsally, in Sc + R_1 from post-basal to medial area	850	207.6	22.5	Oblong with rounded apex and obtuse base	Irregularly sized	Unevenly spaced	220	116.5	5.5	Obovate with truncate apex and auriculate base	Regularly sized	Evenly spaced
Anteos menippe	HW: Dorsally, in Sc + R_1 from post-basal to sub-medial area	600	198.4	8.8	Oblong with rounded apex and obtuse base	Regularly sized, rounded	Evenly spaced	270	113.9	6.7	Obovate with truncate apex and auriculate base	Regularly sized	Evenly spaced
Phoebis argante	HW: Dorsally, on the submedial area of Sc + R_1 and Rs.	680	Not measured		Obovate with rounded apex and auriculate base	Very small, regularly sized	Evenly spaced	300	86.1	3.1	Obovate with rounded or truncate apex and auriculate base	Absent	Absent
Phoebis philea	FW: Ventrally, in CuA_2, on submedial area HW: Dorsally, in Sc + R_1 from post-basal to submedial area	650	127.7	13.3	Oblong with slightly rounded apex and obtuse base	Regularly sized on the distal half. Considerably larger and irregularly sized on the proximal half	Unevenly spaced, much more prominent on the proximal half	350	94.3	2.5	Oblong with truncate apex and auriculate base	Regularly sized	Evenly spaced
Phoebis marcellina	FW: Ventrally, in CuA_2 from post-basal to submedial area HW: Dorsally, in Sc + R_1 from post-basal to submedial area	570	81.9	4.5	Oblong with rounded apex and obtuse base	Regularly sized	Evenly spaced	380	84.8	3.6	Oblong, but wider than the androconial ones, with truncate apex and auriculate base	Absent	Absent
Phoebis statira	FW: Ventrally in CuA_2 from post-basal to sub-medial area	650	117.5	4.6	Ovate, with distal 1/5 abruptly straightening towards the apex, which is acute. Truncate base	Variable length, the ones on the centre may reach approximately 1/2 the total length of the scale	Unevenly spaced	300	80.9	8.5	Ovoid with asymmetrical margins and pointy apex. Pedicel dislocated to the right of median line	Very small, rounded	Rudimentary or absent. Ridges oblique and slightly curved
	HW: Dorsally, in Sc + R_1 from post-basal to sub-medial area							400	86.5/60.1	7.7/3.5	Obovate with truncate apex and obtuse base	Regularly sized	Evenly spaced

†Socket of the androconial scales is involved by a swollen basal area of the scale, which covers the pedicel in lateral view

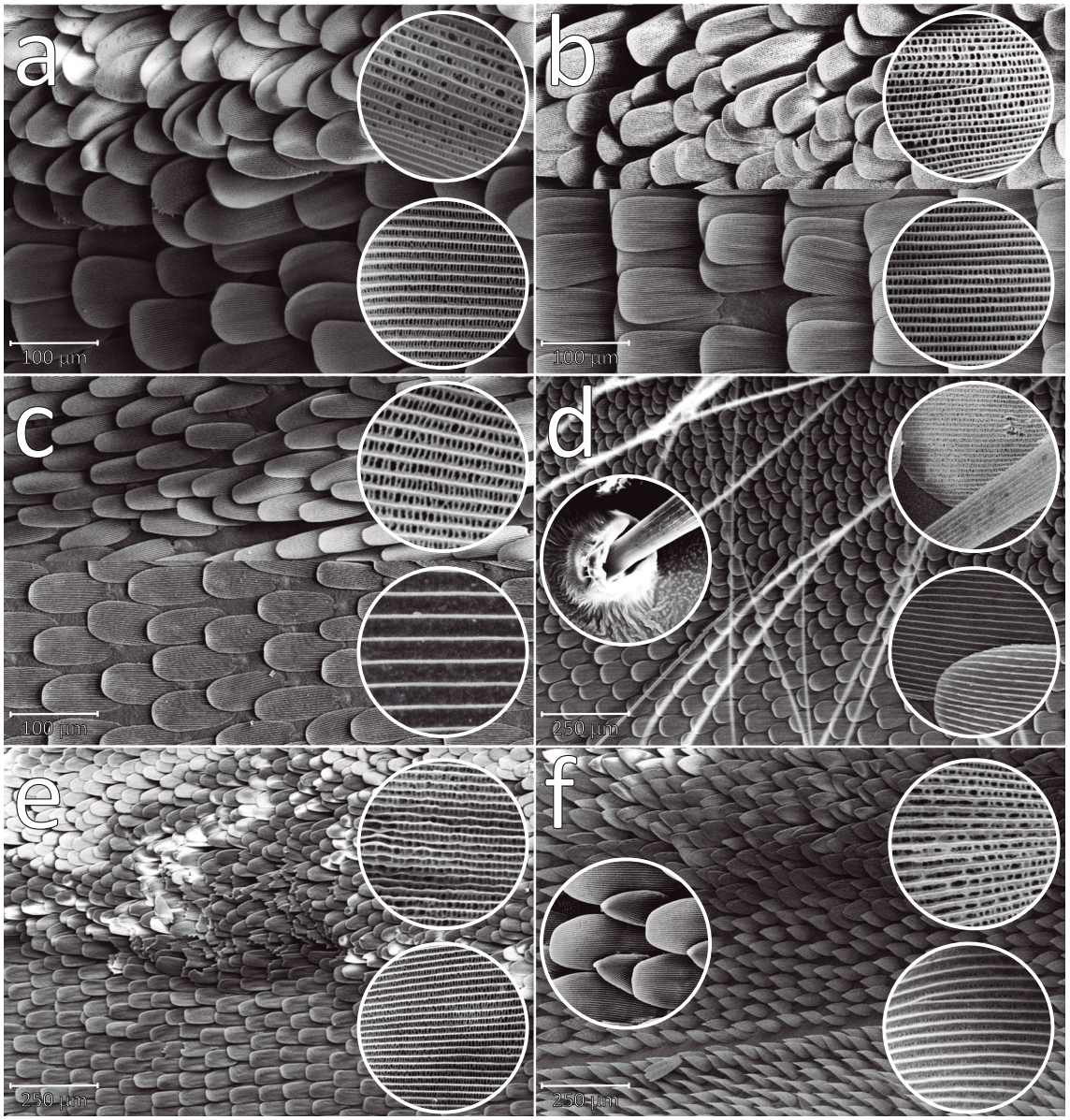

Fig. 2 SEM photographs of wing scales of the *Colias*-clade butterflies of northeastern Brazil. The androconial scales are on the upper portion of the images and the ordinary scales, on the lower portion. At × 500 magnification: (a) *Anteos clorinde*, (b) *A. menippe*, (c) *Phoebis marcellina*; at × 200 magnification: (d) *P. argante*, (e) *P. philea,* (f) *P. statira*. Details of the scales (circles to the right), showing the *striae*, crossribs and windows are at 2.5 k magnification, with exception of (c) and (e), at 5 k magnification. Details on circles to the left: (d) differentiated socket of hair at 5 k magnification and (f) dissimilar shapes of ordinary scales at 1.5 k magnification

surrounding ordinary scales (Fig. 1) and contrarily to the latter, the morphology of androconial scales is relatively constant intraspecifically. Androconial scales are 1.5 to 4 times more densely arranged in the wing surface than the ordinary scales (Table 1, Fig. 2) and inserted in the wing membrane at wider angles (not measured). The windows (perforations) and the respective crossribs on the upper lamella are absent or less evident in the periphery of the scales and are usually less evenly arranged than that of the ordinary scales (Table 1, Fig. 2).

Androconial secretions chemistry

A total of 55 compounds were found exclusively in the androconial extracts of the six species, of which 41 were autapomorphic (Table 2, Supplementary table S2). The most complex androconial chemical profile was obtained from *Phoebis philea*, with the highest number of both overall and species-exclusive compounds (23 and 14, respectively). It also presented the highest number of compounds with high RIs (suggesting lower volatility), and it is in this amplitude that

Table 2 Total relative amounts of dominant compounds (≥ 10% relative percentage in any sample) identified in androconial extracts of males of the *Colias*-clade butterflies (genera *Anteos* and *Phoebis*) of northeastern Brazil. Values between brackets indicate the number of analysed samples in which each compound was present, per investigated species

Kovats index	Compound	*A. menippe* (*N* = 10)	*A. clorinde* (*N* = 8)	*P. statira* (*N* = 9)	*P. marcellina* (*N* = 10)	*P. philea* (*N* = 12)	*P. argante* (*N* = 5)
1853	6,10,14-Trimethyl-pentadecan-2-ol	–	–	–	–	–	88.96 [5]
1892	Benzyl salicylate	9.94 [10]	–	0.45 [3]	37.12 [10]	–	–
1953	Hexadecenoic acid	1.61 [7]	–	0.37 [1]	49.14[10]	0.02 [1]	–
1961	Hexadecanoic acid	2.34 [8]	–	18.55 [8]	–	–	–
2470	Unid. aliphatic ester 2 *m/z*: 96,81,55,97,43	20.08 [10]	–	–	–	–	–
2476	Unassigned compound 1 *m/z*: 85,43,101,84,55	-	–	20.83 [9]	0.17 [2]	4.78 [12]	–
2532	13-Methylpentacosane	-	28.30 [8]	–	–	–	–
2582	Hexenyl octadecanoate	–	15.33 [8]	–	–	–	–
2674	Unid. aliphatic ester 5 *m/z*: 96,81,97,55,43	38.15 [10]	50.72 [7]	–	–	–	–
2691	2-Phenylethyl hexadecanoate	–	–	16.66 [9]	–	–	–
2956	Unassigned compound 5 *m/z*: 94,43,95,134,109	–	–	21.95 [8]	–	10.3 [12]	–
3156	Unassigned compound 9 *m/z*: 94,95,135,43,67	–	–	–	–	17.46 [11]	–
3176	Unassigned compound 11 *m/z*: 94,95,43,135,109	–	–	–	–	12.31 [11]	–
3197	Unassigned compound 12 *m/z*: 94,43,134,95,109	–	–	–	–	22.92 [11]	–

the most dominant constituents appear (Table 2; Supplementary table S2). This also occurred in *P. statira*, but the profiles of the androconial extracts of the two other congenerics are rather distinct. Only a few compounds identified in *P. argante* and *P. marcellina* have high RIs, whereas the most dominant ones are much more volatile: in *P. marcellina*, benzyl salicylate, an unassigned hexadecenoic acid, and hexadecanoic acid and in *P. argante*, 6,10,14-trimethyl-pentadecan-2-ol, which comprises ca. 90% of the total blend (Table 2). Androconial solvent extracts of *P. argante* contained the least number of compounds (6) and most restricted molecular weight amplitude (Supplementary table S2); nonetheless, as only five samples were analysed, it is plausible that this species was under sampled.

As a general observation, the chemical blends of androconial extracts from all species revealed few dominant compounds: two or three compounds corresponded to over 50% of the total blend, while a wider array of compounds occurred in lower concentrations (Table 2; Supplementary table S2).

Although there were slight intraspecific variations in the androconial chemical composition, each species exhibited a characteristic chemical profile (p = 0.001, Global-R = 0.999, Bray Curtis-based; p = 0.001, Global-R = 1, Jaccard-based) and the NMDS ordination segregated the samples (or individuals) in a strongly grouped distribution, relative to species (Fig. 3, Supplementary figure S1). Some compounds were found in androconial extracts of more than one species, but only four were dominant (≥ 10%/species) across species: benzyl salicylate in *A. menippe* and *P. marcellina*; hexadecanoic acid in *P. statira* and *P. marcellina*; an unidentified compound (unassigned compound 5) in *P. statira* and *P. philea* and an unidentified aliphatic ester (unid. aliphatic ester 5) in both species of *Anteos* (Table 2). These co-occurrences of dominant compounds heavily influenced the clustering pattern, as evidenced by the comparison of presence/absence to the relative abundances data (Supplementary figure S2). The Bray Curtis analysis yielded higher similarities among males of the two species of *Anteos*; the males of *A. clorinde* did not even form a cluster of their own, but were rather clustered together with *A. menippe* (Fig. 4, Supplementary figure S2 - A). Furthermore, higher similarities were obtained between *P. marcellina* and *Anteos* spp. than between the former and its congenerics (Fig. 4). Although some dominant compounds co-occurred in two, sometimes three of the investigated species of *Phoebis* (Table 2, Supplementary table S1), no androconial chemical synapomorphy was identified for the genus as a whole. The four species in our study presented very low similarity values, and the chemical blend of *P. argante* actually only shared a single minor compound—linoleic acid—with congenerics (Table 2; Supplementary table S2). As result, all samples

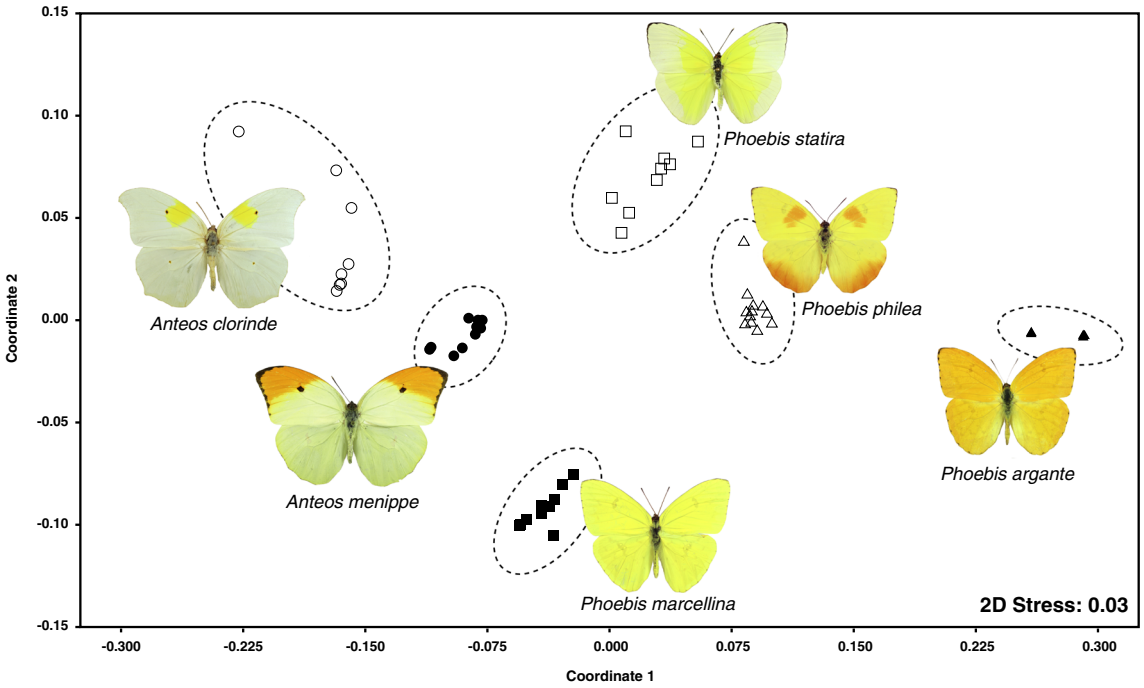

Fig. 3 Bray Curtis-based NMDS ordination for the chemical composition of androconial secretions of the *Colias*-clade butterflies of northeastern Brazil

of *P. argante* formed an outgroup distant from the remainder sulphurs (Fig. 4, Supplementary figure S1). The Mantel tests revealed moderate positive correlations between genetic distances and chemical dissimilarities of the group (r = 0,593, p = 0.001, Bray Curtis-based; r = 0.509, p = 0.001, Jaccard-based) (Fig. 5, Supplementary figure S3).

Discussion

Morphology of androconial scales

The overall morphology of the wing scales of the six investigated species is typical of higher Lepidoptera: hollow structures that contain numerous striae and transversal crossribs that delimit perforations along the upper lamella (Kristensen 1970). The androconial patches, however, are clearly differentiated from the typical scales of the surrounding wing areas by peculiar features such as paler colour, higher density and length of scales and larger windows in the lamella. There is no apparent specialized structure on the wing membrane to protect those patches and reduce volatilization of secretions of the studied butterflies, such as the 'pocket-like cavities' in the Danaini (Boppré 1993) and Ithomiini (Schulz et al. 2004). Nevertheless, the androconial patches of all species are invariably placed on the 'friction areas' of the wings, be it ventrally (post-basal to submedial region of CuA_2) or dorsally (post-basal to medial region of Sc + R_1). These areas remain overlapped when the animal is at rest, a mechanism suggested by Rutowski (1980) as a mean of reducing the volatilization of androconial secretions in species of *Colias.* This indeed seems to be the case of the *Colias*-clade sulphurs, given that there are no androconial patches located in other areas of the wings.

Compared to ordinary scales, androconial scales of the *Colias*-clade butterflies were longer (with the exception of *P. marcellina* and *P. argante*), and more compactly packed, with densities 1.5 to 4 times higher than that of ordinary scales. Similar ratios (≈ 2 androconial: 1 ordinary) were observed in species of *Eurema* and *Colias* (Pieridae: Coliadinae) (Vetter and Rutowski 1979), but not in *Pieris* (Pieridae: Pierinae), in which scent scales are widely distributed on the ventral surface of the wings, with a much lower density than that of ordinary scales (Yoshida et al. 2000). High density of scales may have a complementary effect to the abovementioned overlapping of the wings, as a preventive apparatus to the volatilization of secretions. In fact, most of the area occupied by androconial scales remain concealed by the upper and adjacent scales, with only the distal portions exposed.

The analysis of the micro sculpture patterns also revealed differences between androconial and ordinary scales. More prominent crossribs and larger windows occur in the upper lamella of the androconial scales, especially in the mid portion. The biological significance of the latter trait is not clear, but it may be related to a rapid release of male pheromones during courtship behaviour. According to Barth (1960), a suitable area in which volatilization of the secretion takes place is a distinguishing feature of androconial scales. Thus, by increasing the contact surface, the enlarged windows in a scale-dense region may contribute to the release of

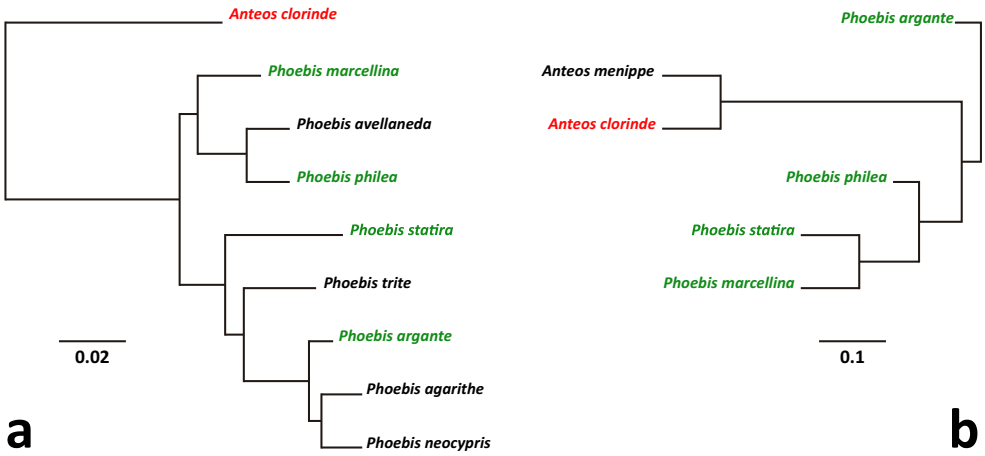

Fig. 4 Comparison between the (**a**) phylogenetic tree adapted from Murillo-Ramos et al. (2018) and (**b**) the dendrogram based on the Bray Curtis similarity index from the androconial chemistry of the *Colias*-clade butterflies of northeastern Brazil

androconial secretions into the air or even facilitate the contact of less volatile compounds with the sensorial surface of conspecific female antennae.

What appeared to be a more specialized structure for releasing pheromonal secretions was found only on the androconia of *P. argante* males, in the form of a dense patch of long hair-like scales. Males of the remaning species lack those hairs on the androconial patches, but present similar features ('fringes') on the anal margin of the forewings (Barth 1960; Bergström and Lundgren 1973), a 'friction area' that is close to the ventral androconia and likely to rub into them during flight or courtship behaviour. Friction of the androconial patches by hovering or buffeting the wings near females is a widespread male behaviour among the Coliadinae (Rutowski 1978; Silberglied and Taylor 1978; Vetter and Rutowski 1979; Kan and Hidaka 1997). So, those fringes might have an analogous function to that of the long hair-like scales of *P. argante* and the potential to act as a facilitator for the release of androconial secretions. This could be considered as a composite system—an area where secretions are produced, associated to a structure that aids their release—as proposed by Barth (1960).

Androconial secretions chemistry

A wide array of compounds exclusive to androconial extracts were found for all investigated species. These chemicals occur precisely in the androconial patches described in this study, which demonstrates that, at least in the wings, their storage and release is restricted to these morphologically specialized areas. Also, we have shown that the composition of androconial secretions is species-specific, suggesting that these compounds take part in intraspecific chemical communication. Behavioural experiments have consistently demonstrated that certain male wing secretions function as sex pheromones in butterflies (Constanzo and Monteiro 2007; Darragh et al. 2017) including Pieridae (Taylor 1973; Silberglied and Taylor 1978; Grula et al. 1980; Rutowski 1980; Kan and

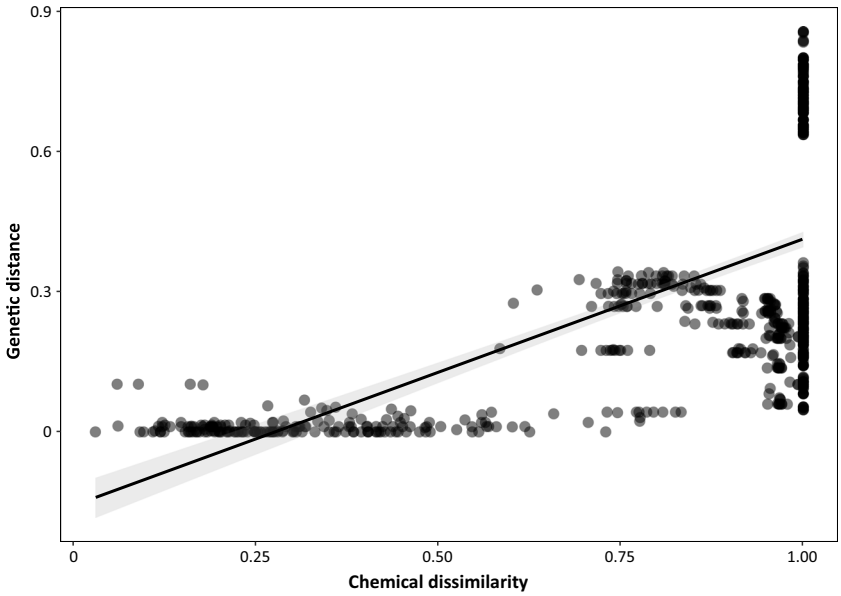

Fig. 5 Correlation between genetic distance and Bray Curtis-based androconial chemical dissimilarity of the *Colias*-clade butterflies of northeastern Brazil

Hidaka 1997; Andersson et al. 2007; Yildizhan et al. 2009). Although we did not conduct such experiments, the absence of androconial constituents from the wings of conspecific females offers compelling evidence of their involvement in mate recognition and/or attraction.

Our results revealed distinct chemical profiles for each investigated species of sulphur. Among butterflies, the degree of chemical differentiation of androconial profiles seems to vary case by case, as shown in studies with sympatric Nearctic and Palearctic Pieridae. *Colias philodice* and *C. eurytheme* Boisduval, 1852 present significant qualitative compositional differences (Grula et al. 1980), whereas for the closely related *Pieris melete* Ménétriés, 1857 and *P. napi* Linaeus, 1758, very similar androconial chemical profiles were evidenced. In this later case, marked dissimilarities were restricted to the concentrations of two stereoisomeric monoterpenes (neral and geranial) and a species-specific presence of another (linalool) (Hayashi et al. 1977). Among the species in our study, large qualitative and quantitative chemical differences were observed, which reinforces the highly species-specific character of the constituents in androconial secretions. This strong differentiation of sex-related traits, if properly recognised by the females, may act alongside the also very distinct species-specific male colour patterns, leading to a robust pre-zygotic isolation mechanism. The complementary use of visual and chemical signs to recognise conspecific males is documented for female butterflies (Silberglied and Taylor 1978; Constanzo and Monteiro 2007). Nevertheless, major differentiation of chemical profiles per se does not necessarily imply a more efficient mechanism of sexual isolation. Female butterflies may rely on different compound concentrations or on the presence of a few or even single constituents within the total androconial chemical blend of conspecific males for mate recognition (Hayashi et al. 1977; Yildizhan et al. 2009). Further investigation is required to elucidate the roles of individual compounds and their blends in the behaviour of the *Colias*-clade sulphurs.

The most diverse androconial blend among the species in our study belongs to *Phoebis philea*. It presents a dominance of lowly volatile compounds (those eluting later than pentacosane on a non-polar GC column), which may be a clue to behavioural aspects of courtship of this species. If in fact those compounds are involved as semiochemicals in mate recognition and quality assessment, we could expect substantial androconia-antennae contact between males and females. This behaviour was indeed reported for *P. marcellina* (Rutowski 1983), in whose androconial secretions we identified a predominance of more volatile compounds than those of *P. philea*. Presumed lowly volatile pheromones are also found in other species of Pieridae (Rutowski 1980; Grula et al. 1980; Sappington and Taylor 1990) and Danainae (Meinwald et al. 1969; Meinwald et al. 1974; Schulz et al. 1993), in which direct contact occurs during courtship (Pliske and Eisner 1969). On the other hand, high volatile compounds were characteristic of particular species such as limonene in *P. marcellina* and farnesene in *A. menippe* and *P. marcellina*. It is believed that butterflies also use volatiles as mating cues at close-range communication (Boppré 1984). Therefore, such volatiles may not be negligible just because of their low concentrations in relation to the major compounds.

Few of the major compounds found in our study were previously reported in butterflies: 13-methylpentacosane as a contact sex pheromone in the sulphur *Colias philodice* (Grula et al. 1980), hexadecanoic acid in the male hairpencils of many species of Danaini (Schulz et al. 1988; Schulz et al. 1993) and 2-phenylethyl hexadecanoate as a major androconial compound in the nymphalid genus *Bicyclus* (Wang et al. 2014). On the other hand, many compounds we identified in sulphurs were previously unknown from Pieridae and Lepidoptera. For minor blend constituents, this may be due to advances achieved in the analytical methodology when compared to the results of previous exploratory works on the androconial chemistry of Pieridae, which date to ca. 40 years ago (Hayashi et al. 1977; Kuwahara 1979; Grula et al. 1980; Honda and Kawatoko 1982). Nonetheless, in the case of major constituents, because we are dealing with species of a poorly investigated Neotropical clade from a chemistry standpoint, a number of novelties were arguably expected to occur. For example, as far as we are aware, benzyl salicylate has not been recorded as an lepidopteran semiochemical to date. It is nonetheless reported as a male sex pheromone component of the Florida woods cockroach (*Eurycotis floridana* Walker, 1868; Farine et al. 1994) and a constituent in floral scents across different families of angiosperms (Knudsen et al. 2006). It is present in three of the six *Colias*-clade species in our investigation, being a major constituent in the androconial secretions of *A. menippe* and dominant in *P. marcellina*. The compound has fixative properties (Sturm and Peters 2007), which could arguably interfere with the volatility and tenacity of other compounds in the androconial blends.

When compared to recent phylogenies of the group (Wahlberg et al. 2014; Murillo-Ramos et al. 2018; Núñez et al. 2020), the chemical similarity of the androconial blends reflected the phylogenetic proximity between the two investigated species of *Anteos*. However, neither the phylogenetic relationships among the four species of *Phoebis* nor between the two genera were supported. When treated as a whole, a moderate correlation between genetic distance and chemical dissimilarity was in fact noticed for the group, but aside the co-occurrence of a dominant unidentified aliphatic ester between the species of *Anteos*, assumed to be an informative synapomorphy, no such feature occurred among *Phoebis*. Because we are dealing with elements directly involved in mating and species recognition, a strong differentiation in such features is expected among closely related species in order to ensure reproductive isolation. Therefore, our results did not yield evidence of a conclusive phylogenetic signal on

the chemical composition of androconial secretions for the *Colias*-clade butterflies.

Conclusions and perspectives

The investigation on the scale morphology of males evidenced androconial patches that are clearly differentiated from the non-androconial wing surface and exhibit particular features that could facilitate the release of semiochemicals, such as higher density of scales, higher length and larger windows in the upper lamellae, in comparison to the ordinary scales. Furthermore, the analyses of the androconial extracts of all six studied species revealed a wide array of species- and gender-exclusive compounds—some of which novel for Lepidoptera. The species-specific nature of the blends of the sympatric and ecologically similar species point towards the value of such diversification in mating-oriented strategies of butterflies. Nevertheless, a number of questions arose from our results: are all the androconial chemical components involved in sexual interactions or are some rather cues used in male-male communication? Are they directed exclusively to mate recognition or are there compounds that indicate male fitness/quality? Do they act as a backup for visual stimuli or do they provide distinct information? And, on which sensory cues, visual or chemical, do female butterflies rely more strongly? Some of these questions were assessed for Neartic pierids, and conclusions varied among species. Very diverse and species-specific androconial chemical blends occur in the *Colias*-clade sulphurs of northeastern Brazil; we expect that a wide array of different strategies are involved in their sex-oriented chemical communication as well.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13127-020-00475-8.

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Author contributions Carlos Eduardo B. Nobre conceived, designed and performed the research, collected and analysed the data, contributed with reagents and materials, prepared figures and tables, authored the drafts of the paper, approved the final draft.

Layse A. S. Lucas contributed with analysis tools, analysed the data, reviewed the drafts of the paper and approved the final draft.

Rafael J. R. Padilha contributed with materials and analysis, prepared figures and approved the final draft.

Daniela M. A. F. Navarro contributed with reagents, materials and analysis tools, and approved the final draft.

Luiz C. Alves contributed with reagents, materials and equipment, and approved the final draft.

Artur C. D. Maia conceived and designed the research, analysed the data, contributed with reagents and materials, edited figures, reviewed the drafts of the paper and approved the final draft.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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