Chemical Cues in Species Recognition and Reproductive Isolation of *Tetragnatha* Spiders (Araneae: Tetragnathidae)

By

Seira Ashley Adams

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Abstract

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The mechanisms by which reproductive isolation evolves and is maintained between populations is fundamental to our understanding of the evolution and accumulation of biodiversity. Studies on the role of visual and auditory cues have provided key insights into the mechanisms of divergence and reproductive isolation. Yet, major questions remain, in particular in situations where there is little visual or acoustic evidence of courtship or any other means of mate recognition. Chemical cues are another, arguably the most widespread, modalities of communication, yet their importance in species recognition and reproductive isolation remains largely unknown. To date, the role of chemical cues in arthropod communication has emphasized agricultural pests, creating a gap in our understanding of their potential role in reproductive isolation in other arthropod groups and, more specifically, what compounds are involved in these processes. My dissertation focuses on the role of chemical cues in species recognition in long-jawed spiders in the genus *Tetragnatha* to investigate whether: 1) male *Tetragnatha* spiders use chemical cues for species recognition and mate choice, 2) chemical cues play a role in the species recognition and adaptive radiation of Hawaiian *Tetragnatha* spiders, and 3) specific chemicals found on the cuticle of Hawaiian *Tetragnatha* spiders serve a role in both ecological divergence and sexual divergence in the form of desiccation resistance and mate recognition.

Compared to the commonly studied arthropods in chemical research, spiders differ considerably in lifestyle and behavior. Although many behavioral studies have clearly demonstrated the use of chemical cues in spider communication, specifically in mate attraction, the extent to which these chemical cues play a role in species recognition and reproductive isolation is unknown. To date, sex pheromones have been identified and tested in only six spider families despite the immense abundance and diversity of spiders globally. *Tetragnatha* spiders, in particular, are distributed worldwide, typically building orb webs over water. These spiders are ideal for studying the use of chemical cues in reproductive isolation as they are exclusively nocturnal and have a mating strategy that involves the locking of jaws with little evidence of either visual or auditory recognition cues. Furthermore, *Tetragnatha* spiders have adaptively radiated in Hawaii to form over 50 different species and is one of the most well-studied arthropod adaptive radiation to date.

The work presented here utilizes this system to first (**Chapter 1**) determine whether chemical cues are used by male *Tetragntha* spiders for species recognition using a combination of behavioral assays and chemical analysis to identify the behavioral response and chemical compounds involved in this recognition. The results provided clear behavioral and chemical evidence that males use methyl ether compounds found on the silk of female spiders for species recognition. I then apply the same methods to (**Chapter 2**) investigate the role of chemical species recognition in the adaptive radiation of Hawaiian *Tetragnatha* spiders, focusing on sites where multiple species co-occur. Results show that, at a site where 8 close relative co-occur, the chemical cues of each species is distinct, with no overlap between species. Moreover, populations of a given species can differ in their chemistry, depending on the set of species with which they co-occur. The inference is that chemical cues are likely candidates in facilitating rapid reproductive isolation and speciation in this lineage. Lastly, (**Chapter 3**) I investigate whether chemicals found on the cuticle of Hawaiian *Tetragnatha* spiders play a part in both ecological and sexual divergence through the roles of desiccation resistance and mate recognition. Focusing of a species that lives in an almost desert-like habitat on Hawaii and their close relatives that live in wet forest, I measure the desiccation resistance of these species and then quantify the cuticular chemicals. Results indicate that the methyl ether compounds used for mate recognition does not correspond with the desiccation resistance of the species while a different set of compounds, the cuticular hydrocarbons, do. Thus, the two roles of desiccation resistance and mate recognition are regulated by separate classes of chemicals.

Combined, these three chapters provide unprecedented insights into the rarely studied role of chemical cues in reproductive isolation and speciation within a rapidly diversifying lineage of spiders, and pave the way for detailed studies on the relative timing of natural and sexual selection in facilitating adaptive diversification.

To all the spiders…

Thank you greatly for your contributions

And to my dog

Link

You're too cute for your own good

May you live long and prosper

Contents

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Chapter 1

Chemical Species Recognition in a *Tetragnatha* Spider (Araneae: Tetragnathidae)

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Previously published material

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Abstract

Much of our knowledge regarding the role of chemicals in species recognition in arthropods is based on a few taxonomic groups, predominantly insect pest species. To investigate the chemical underpinnings of species recognition cues in other arthropods, we conducted mate choice experiments and analyzed the chemical profiles of two species in the long-jawed spider genus *Tetragnatha* from allopatric populations across two different continents. In two separate bioassays, in which male *T. extensa* spiders were presented with either web silk or extracts from the silk of conspecific and heterospecific females, males consistently chose the silk or silk extract of conspecific females over those of heterospecifics. We examined the chemistry affecting this response using gas chromatography/mass spectrometry to analyze silk and whole-body extracts of the spiders. The major compounds in the extracts were identified as long chain aliphatic methyl ethers. The chemical profiles of the two species differed: the *T. extensa* profile consisted of 12,20-dimethylnonacosyl methyl ether (**A**), 8,14,20-trimethylnonacosyl methyl ether (**B**), and 6,14,20-trimethylnonacosyl methyl ether (**C**), while the profile of *T. versicolor* consisted of **B** and 14,20-dimethylnonacosyl methyl ether (**D**). Within each species, chemical profiles of females and males did not differ. Our results suggest that these methyl ethers are involved in species recognition of *Tetragnatha* spiders. This is the first study to propose compounds involved in species recognition in spiders.

Introduction

The chemical sense is the major mode of communication in many organisms, especially for attracting and distinguishing conspecific (Ache and Young 2005; Dethier 1990; Smadja and Butlin 2009; Strausfeld and Hildebrand 1999). Numerous studies have led to the characterization of over 3000 compounds that play crucial roles in species recognition and mate assessment (Symonds and Elgar 2008). The role of sex pheromones has been well studied in moths, beetles, Hymenoptera, and *Drosophila* flies, with volatile chemicals and cuticular hydrocarbons commonly used for attracting and assessing mates (Blomquist and Bagnères 2010; Francke and Schulz 2010). However, historical emphasis on agricultural pest insects has created a gap in our understanding of how some arthropod groups use pheromones, and more specifically, what compounds are used. The current study seeks to fill this gap by examining the species specificity of pheromones in spiders and determining how they are used in communicating identity. Spiders differ considerably in lifestyle and behavior from many of the insects studied for chemical communication. While studies on spiders have demonstrated the widespread use of chemosensory information in communication, particularly mate attraction (see reviews in Fischer 2019; Gaskett 2007; Schulz 2004; Schulz 2013; Trabalon and Bagnères 2010; Uhl 2013; Uhl and Elias 2011), the extent to which these pheromones communicate species identity is unknown. To date, sex pheromones have been identified and synthetic compounds tested in only six spider families (Chinta et al. 2010; Fischer 2019; Schulz 2013; Schulz and Toft 1993a; Tichy et al. 2001; Trabalon 2013). The species specificity of chemical information has been investigated behaviorally in many different spider groups, especially on cursorial taxa (Cerveira and Jackson 2013; Cross and Jackson 2013; Nelson et al. 2012 on salticids, Roland 1983 on agelenids, Barth and Schmitt 1991; Stratton and Uetz 1983 on ctenids, Roberts and Uetz 2004a, Roberts and Uetz 2004b on lycosids, Costa-Schmidt and Machado 2012 on trachaleids). However, the results have been mixed, leading several authors to conclude that, although chemicals may play an important role in the mating behavior of spiders, other modalities such as visual and vibrational are more critical in species recognition, particularly in cursorial spiders that have acute vision and elaborate vibratory communication (e.g., Barth and Schmitt 1991; Costa-Schmidt and Machado 2012; Roberts and Uetz 2004a; Roberts and Uetz 2004b; Uetz and Roberts 2002). Web-building spiders, on the other hand, are sit-and-wait predators with limited visual capacity, suggesting that both chemical and vibratory signals may play a major role in species recognition. Indeed, both olfactometer and contact behavioral studies on web-building theridiids in the genus *Latrodectus* show that some species discriminate conspecific from heterospecific individuals based on web silk (Baruffaldi 2016; Kasumovic and Andrade 2004; Jerhot et al. 2010; Scott et al. 2015). Furthermore, behavioral assays testing the response of males to female silk of two linyphiids in the genus *Linyphia* found reduced male response when contacting heterospecific female webs (Schulz and Toft 1993a). The current study set out to ascertain the use of species-specific pheromones in two species of long-jawed orb-weaving spiders in the genus *Tetragnatha* (Tetragnathidae), and to characterize the chemical nature of any specificity.

Tetragnatha spiders occur worldwide. They build loose, fragile orb webs over bodies of water and are primarily nocturnal (Danielson-François et al. 2002; Gillespie 1987; Levi 1981). As with most spiders, males become itinerant once they reach sexual maturity, and congregate around the webs of females (Danielson-François et al. 2002). Courtship rituals in most spiders involve extensive visual or vibrational communication (Uetz and Stratton 1983; Uhl and Elias 2011);

however, courtship of *Tetragnatha* spiders is much less elaborate. Males arriving at the edge of a female's web rush toward the female with open chelicerae after a female signals receptivity, at which point the two then lock chelicerae and mate (Danielson-François et al. 2002; Kaston 1948). Given the lack of any obvious visual or vibrational communication, the extent to which either sex exhibits mate choice is not clear with, potentially, coupling entirely due to the mechanical locking of chelicerae (Levi 1981). However, it is also possible that pheromones play a role in mate choice.

While the role of chemical cues in *Tetragnatha* spiders is largely unknown, pheromones are suggested or known to play a role in mate choice in spiders in general. For example, in a tetragnathid in the genus *Metellina* that employs elaborate vibrational signaling during courtship, unlike *Tetragnatha* spiders, behavioral experiments found males assessed and chose mates based on cues related to female silk (Prenter et al. 1994).

Before investigating the details of chemical mate choice in *Tetragnatha* we considered it necessary to investigate first, whether possible silk pheromones elicit a species-specific response. Therefore, we conducted bioassays using the silk and silk extracts of two species: *T. extensa* (Linnaeus, 1758) and *T. versicolor* Walckenaer, 1841. These species are allopatric but not sisters, although they are closely related (Levi 1981). *Tetragnatha extensa* has a wide distribution across the Holarctic region with a broad ecological range (Dondale and Redner 2003; Levi 1981; Nentwig et al. 2020), while *T. versicolor* is found in North America and Central America and does not overlap with *T. extensa* (Dondale and Redner 2003; Levi 1981). Male *T. extensa* were first given the choice between silk threads of conspecific and heterospecific females, and then by controlling for any morphological differences in silk, a choice between silk extracts of females applied to individual cotton threads. We then investigated the chemistry of silk extracts using gas chromatography/mass spectrometry (GC/MS). Given previous work (Schulz 2013), we hypothesized that species-specific recognition signals in the two *Tetragnatha* species should be either volatile compounds or contact-based surface lipids. Such lipids might be hydrocarbons, as often found in insects, but could also include other compound classes, e.g., alkyl methyl ethers, which are abundant and species-specific in several linyphiids and the nephilid *Nephila clavipes* (Linnaeus, 1767) (Schulz 2013) or long chain esters, which show a population-dependent composition in *Anolesimus eximus* (Bagnères et al. 1997). While we could not observe any volatile compounds in extracts, differences in the composition of surface lipids between the species were detected. To characterize individual lipid components, we used chemical microderivatization and GC/MS analyses to propose structures for the most abundant components (alkyl methyl ethers).

Materials and Methods

Study Organisms

Twenty one adult and subadult female and 11 male spiders of *Tetragnatha versicolor* Walckenaer, 1841 were collected from plants on the banks of Eel River in Angelo Coast Range Reserve in Brandscomb, California during the summer of 2016, and brought to the University of

Greifswald, Germany, in 3 cm diam. Vials. In Greifswald, *T. versicolor* individuals were transferred to larger 10 cm high, 5 cm diam. Vials with a sponge plugging the bottom, a gauze top, and a stick inside for spiders to climb. The sponge was constantly soaked with water to maintain humidity. In the same summer, 35 adult and subadult *T. extensa* (Linnaeus, 1758) were collected from around a pond 2 km south of Greifswald, Germany and brought into the laboratory to be housed individually in the same manner as *T. versicolor*. Spiders were kept under a natural light regime and fed twice a week with approximately six flightless *Drosophila hydei*. Spiders were checked every morning to record molting or egg-laying. Spiders were labelled as unmated if they molted to maturity in the lab, and mated if they were already mature when caught in the field (where population densities are high).

Female spiders were moved into a 28 × 28 × 9 cm plastic frame for web construction the day before webs were used for behavioral experiments or chemical extraction. When storing frames with spiders inside, the open sides were covered with a plastic spacer with Vaseline to prevent spiders from escaping; it also allowed spiders to attach silk to the surface. A sponge base holding two bamboo sticks standing vertically was placed inside the frame to provide more structure and silk anchoring points. A 12 × 7 cm plastic container filled with water was placed inside each frame for humidity. Each spider was provided with live mosquitoes collected from the field, which stimulated web building more readily than did the diet of flightless *Drosophila*. Spiders built orb webs overnight and the freshly built webs were used in tests the following morning.

Web Silk Choice Trials

To assess whether *T. extensa* males discriminate conspecific females through silk, a strand of silk from the web of female T. extensa and one from that of *T. versicolor* were presented to males in a silk-choice trial ("silk trials", Fig. 1a). One female *T. extensa* and one female *T. versicolor* with complete orb webs were chosen randomly for each trial. Before the webs were manipulated for trials, a brush was used to remove females from their webs without touching the web itself. Females were stored in individual vials and released back into their webs at the end of each trial. Once females were removed from the webs, the frames with webs were positioned side by side. The position of the web of each species was alternated between trials. In each web, a single radial silk strand was used so that the two silk strands could be easily pulled toward each other without breaking the strands. To free the selected radial strand from the body of the web, the spiral strands that crossed and connected to the radial strand were carefully cut away using clean dissection scissors. The radial strand was then be pulled outward and away from the web and attached to the end of a 25 cm-long bamboo stick. The bamboo stick was held horizontally 10 cm off the ground by a stand and clamps. The same procedure was repeated with the other web until two radial strands were attached to the bamboo stick in a Y-formation at a slight upward angle of about 20° from the stick to the hub of the webs. All tools used were washed with 70% ethanol before and after every procedure conducted on a web.

Once the webs of the two species were set up with silk strands attached to the bamboo stick, a *T. extensa* male was randomly selected and placed gently, using a brush, facing the web on the opposite end of the bamboo stick. All males initially stretched their legs out in a stick-like formation and clung to the bamboo stick without moving (a typical *Tetragnatha* behavior when in resting/hiding position). If the male did not move from this position within 30 min after being

placing onto the stick, it was removed gently and replaced with a new male. Males were only used once in experiments. The bamboo stick was cleaned with 70% ethanol only if a male moved from the starting position up the stick. Males that moved within 30 min all walked up the stick toward the web and contacted the two silk strands at the end. A male would then touch the two silk strands with the first and (often) second pair of legs. A male was considered to have made a choice when it moved off the stick and walked up one of the silk strands. All trials were recorded using an iPhone 6 camera (Apple Inc., Cupertino, CA). Videos were checked to see if males touched both silk strands, which was the case in 16 of 19 (84%) silk trials. Only trials in which a male touched both silk strands were included in the analysis.

Silk Extract Choice Trials

To assess whether *T. extensa* males discriminate conspecific from heterospecific females using chemical cues alone, chemicals from the webs of female *T. extensa* and *T. versicolor* were extracted with dichloromethane (DCM) and presented to males on thin cotton threads (Gütermann basting thread) in a choice trial ("silk extract trials"). The application of silk extracts on cotton threads allowed for the separation of chemical cues from that of haptic cues, such as silk diameter and/or structure. To prepare silk extracts, freshly built, complete orb webs of female *T. extensa* and *T. versicolor* spiders were selected in the morning before a trial. All available silk from each web was wrapped around a clean metal spatula. Clean tweezers were used to push the wrapped-up silk into a tight ball, which was then placed in a 2 ml glass vial containing 0.2 ml of DCM. After 30 min, the silk ball was removed, and the extract used in the trials on the same day. To mimic the Y-formation of the silk strands used in the silk trials, two pieces of cotton thread (15 cm long) were attached to the end of the horizontal bamboo stick by masking tape, and then stretched out and attached to a separate vertical bamboo stick held upright by the sponge base so that the two threads were spread out into a Y-formation (Fig. 1b). The cotton threads sloped upward at about 20° from the horizontal bamboo stick. Silk extracts of either female *T. extensa* or *T. versicolor* web were dispensed onto each cotton thread. The side of the extract of each species was alternated in successive trials. Extracts were left for 1 min, allowing DCM to evaporate. Then, a randomly selected male *T. extensa* was placed gently at the opposite end of the bamboo stick in the same manner as for the silk trials. A male was considered to have made a choice when it moved off the bamboo stick and walked up one of the cotton threads. A total of 18 silk extract trials were conducted. All trials were recorded using an iPhone 6 camera. Only males that touched both cotton threads (N = 16, 89%) were used in the analysis. New cotton threads were used for each trial and all tools cleaned with 70% ethanol before and after each trial.

Chemical Analysis of Spider Extracts

Web and whole-body extracts of *Tetragnatha* spiders were analyzed by GC/MS (Agilent 7890A/5975C). To minimize chemical contamination, oven bags (Toppits brand) known to emit only low amounts of volatile organic compounds (VOCs) were used in place of Vaseline for the walls of the frames where the spiders were stored and left to build webs. Freshly made complete orb webs were extracted in the morning (see above) and the extract stored at 20 °C until chemical analysis. For whole body extractions, spiders were first anesthetized with CO_2 and then transferred into a 2 ml glass vial containing DCM. The legs were folded so that the entire body

was submerged in the solvent and extracted for 30 min. Because of the size difference, 1 ml of DCM was used to extract the larger *T. versicolor* spider extractions while 0.7 mL of DCM was used for *T. extensa*. After extraction, spiders were stored in 70% ethanol, while extracts were stored at −20 °C. All tools were washed and cleaned in DCM before and after each step of extraction.

DCM extracts of silk and whole-body extracts were analyzed by GC/MS (electron impact; 70 eV). Web extracts from 4 mated and 3 unmated females of *T. versicolor*, as well as 3 mated and 2 unmated females of *T. extensa* were analyzed. In addition, pooled extracts of 5 mated and 5 unmated females of both species were made and analyzed. For whole body extracts, 5 mated female, 5 unmated female, 5 mated male, and 4 unmated male *T. versicolor* extracts, as well as 4 mated female, 5 unmated female, 4 mated male, and 4 unmated male *T. extensa* extracts were analyzed. The GC was fitted with a HP-5 MS column (Agilent, 30 m length, 0.25 mm ID, 0.25 µm film thickness) with helium as carrier gas. Extracts were analyzed in the splitless mode, with a column oven temperature program of 50 °C for 5 min, increased by 5 °C.min^{-1} to 320 °C. Injector and transfer line temperatures were maintained at 250 °C.

Microreactions

For the determination of methyl branch positions of long-chain alkyl methyl ethers, extracts were derivatized by microreactions (Schulz 1997). Oxidation using ruthenium tetroxide converted methyl ethers into the corresponding methyl esters (Schulz and Toft 1993b). A catalytic amount of ruthenium tetroxide is generated in situ from ruthenium(III) chloride and sodium periodate. Ruthenium(III) chloride (1 mg) was added to a 1.5 ml GC vial containing natural extract (10 µl), CCl_4 (50 µl), acetonitrile (50 µl), water (75 µl), and sodium periodate (10 mg). The reaction mixture was stirred vigorously at room temperature for 2 h. The aqueous phase was extracted with DCM (3 × 200 µl), and the organic phases combined and diluted with diethyl ether (500 µl) and dried by elution through a small pipette filled with sodium sulfate. The pipette was rinsed with diethyl ether (3 × 200 µl) and the combined solution filtered again through a pipette with a small amount of silica gel that was again rinsed with diethyl ether (3 × 200 µl). The solvent was removed from the final combined solution by a stream of nitrogen and redissolved in DCM (10 µl). This solution was used for GC/MS analysis.

To obtain the corresponding nitriles, methyl ethers were first converted into iodides using trimethylsilyl iodide (Schulz 1997). Reaction of the iodides with tetraethylammonium cyanide in DCM yielded the target nitriles. To convert methyl ethers into iodides, trimethylsilyl iodide (4 µl) was added to the natural extract (10 µl) dissolved in DCM (20µl) in a 1.5 ml GC vial. The vial was placed in a heating block at 60 °C for 2 h and shaken intermittently. The reaction was stopped by addition of saturated sodium bicarbonate solution (40 µl), and the mixture extracted with DCM (3 × 40 µl). The organic phases were separated from the aqueous phase and collected in a GC vial. Sodium chloride (3 mg) was added to dry the solution. The solution was removed using a pipette and the remaining sodium chloride washed with DCM (3 × 40 µl). The combined solutions were placed into a new GC vial with a stir bar, and tetraethylammonium cyanide (1 mg) added. The reaction mixture was stirred at room temperature for 16 h. Addition of water (200 µl) and vigorous stirring for 10 min stopped the reaction. The aqueous phase was removed using a pipette. The solvent was removed under a stream of nitrogen and the residue triturated

with pentane (3 × 100 µl). Pentane extracts were combined, and the solvent removed by a stream of nitrogen. The residue was dissolved in DCM (10 µl), ready to be analyzed by GC/MS.

Results

Web Silk Trials & Silk Extract Trials

In the silk trials, *T. extensa* males walked up the silk strand of the web of a conspecific female at a higher probability than for the silk strand of the heterospecific female ($X^2 = 12.25$, df = 1, $P = 0.005$; 15:1) (Fig. 1a, Fig. 2). This was also the case in the silk extract trials, in which T. extensa males walked up the cotton thread with the conspecific web extract at a higher probability than up the thread with heterospecific web extract ($X^2 = 12.25$, df = 1, $P = 0.005$; 15:1) (Figs. 1b, Fig. 2).

Chemical Analysis and Identification of Compounds

Silk and cuticular DCM extracts of mated and unmated males and females of *T. extensa* and *T. versicolor* spiders were analyzed by GC/MS. Three major compounds, **A**, **B**, and **C** were found in extracts of *T. extensa* and two, **B** and **D**, in extracts of *T. versicolor*. The total ion chromatograms (TIC) of adult female body extracts are shown in Fig. 3, while TICs of all other sample types can be found in Figs. S1-S4 (Supplementary material). No differences were observed between the sexes nor between mated females and unmated females for the two species. The same compounds were found in both silk and whole-body extracts, in higher quantity in the latter.

All mass spectra of compounds **A**-**D** showed an ion at *m/z* 45 (C_2H_5O), characteristic of either methyl ethers or methyl carbinols. The latter usually has *m/z* 45 as the base peak in the spectrum (Schulz and Toft 1993b); however, this was not the case in these spectra (Fig. 4). Therefore, a methyl ether structure is likely for compounds **A**-**D**. The most intense ion in the higher mass range can be attributed to $[M - 32]^+$, as M^+ ions are usually not visible in the spectra of long-chain methyl ethers (Schulz and Toft 1993b). With $[M - 32]^+$ as the heaviest observable ion, the mass of the compounds was assumed to be 466 and 480,with molecular formulae of $C_{32}H_{66}O$ and $C_{33}H_{68}O$, respectively. Apart from ions characteristic of saturated methyl ethers, the mass spectra indicated, due to non-uniform distributions of ion intensities, the presence of methyl branches, as often found in other cuticular lipids of arthropods. Nevertheless, little information on the position and number of the methyl groups were obtained from the spectra. Therefore, representative samples from each set were derivatized to transform methyl ethers either into corresponding methyl esters or nitriles, allowing determination of the positions of the methyl groups. Methyl esters are especially useful for detection of methyl groups at C-2, C-3, and C-4, difficult to detect via nitriles (Schulz and Toft 1993b; Schulz 1997). However, the spectra revealed the absence of methyl groups in these positions for all compounds.

The ester derivative of lipid A showed a pair of characteristic ions at *m/z* 199 and *m/z* 227, the difference of 28 Da indicating a preferred cleavage on both sides of a methyl group at C-12 (Fig.

5) (Ryhage and Stenhagen 1960). The nitrile derivative showed a pair of ions at *m/z* 306 and *m/z* 334, indicating a methyl branch at C-20, and a second pair at *m/z* 180 and *m/z* 208, confirming the methyl group at C-12 (Schulz 1997). Therefore, the proposed structure of lipid **A** is 12,20-dimethylnonacosyl methyl ether. The experimental gas chromatographic retention index of I 3194 was in good agreement with the value (I_c 3188) calculated according to the increment system we previously developed (Schulz 2001).

In a similar manner, lipid **B** was surmised to be 8,14,20-trimethylnonacosyl methyl ether (I = 3224, I_c = 3221), as *m/z* 143 and 171 of the methyl ester indicated the first methyl group at C-8, and *m/z* 124/152, 222/250, and 320/348 of the nitrile, indicated methyl groups at C-8, C-14, and C-20. In ether **C**, the first methyl group was shifted to C-6 compared to **B**. Its structure, 6,14,20-trimethylnonacosyl methyl ether (I = 3229, I_c = 3228) was elucidated from *m/z* 115/143 and 241/269 of the methyl ester and *m/z* 222/250 and 320/348 of the nitrile. The C-6 methyl group was not clearly assignable by the nitrile derivative, but the methyl ester proved its location. Finally, compound **D** was elucidated as an isomer of **A**, 14,20-dimethylnonacosyl methyl ether (I = 3193, I_c = 3188), indicated by *m/z* 227/255 in the methyl ester spectrum and *m/z* 207/236 and 306/334 in the nitrile spectrum. The calculated and measured retention index values matched in all cases, further supporting our structural assignment.

Discussion

Our study shows that male *T. extensa* use chemical cues to discriminate between the silk of conspecific and heterospecific females. In silk trials, *T. extensa* males preferred silk from the web of conspecific females to silk from *T. versicolor* webs. Likewise, in silk extract trials, *T. extensa* males preferred silk extract of conspecific females to silk extract of *T. versicolor* females. This suggests that the species differ in their pheromones, and demonstrates that species recognition is likely not based on physical properties of silk.

Our results build on a growing body of work showing that male spiders can distinguish and prefer conspecifics over heterospecific females using chemical cues found on female silk. Chemical analyses of the two *Tetragnatha* species in our study demonstrated that web and body extracts of *T. extensa* differed in chemical composition from those of *T. versicolor*, thereby corroborating the behavioral tests. GC/MS analysis of web and body extracts showed that each species has a unique set of compounds, with *T. extensa* profiles comprising 12,20-dimethylnonacosyl methyl ether (**A**), 8,14,20-trimethylnonacosyl methyl ether (**B**), and 6,14,20-trimethylnonacosyl methyl ether (**C**), and *T. versicolor* profiles comprising **B** and 14,20-dimethylnonacosyl methyl ether (**D**). Although compound **B** was common to both species, the relative ratios of compound **B** to the other compounds differed greatly. Extracts from *T. extensa* contained relatively low amounts of **B**, whereas in those of *T. versicolor* it was the major component. This commonality of **B** might be explained by the close relatedness of *T. extensa* and *T. versicolor* (Levi 1981) and/or by these two species not co-occurring. In future work, the chemical profiles of species that co-occur, that are closely and distantly related, as well as different populations of a given species should all be investigated so as to understand better the evolutionary pattern of chemical diversity and species recognition in the genus.

Based on the combined results of the behavioral assays that showed recognition of species identity through chemical cues, and the results of the chemical analysis that showed species-specific differences in chemical composition of spider extracts, we consider the methyl ethers as candidate compounds for species recognition in *T. extensa*. Compared to species-specific cuticular hydrocarbon profiles found in many insects, the profile of *Tetragnatha* spiders were simple, consisting of only a few major compounds that, based on the proposed structures, are likely to have similar physical properties (for example water tightening and fluidity) (Gibbs 1998; Gibbs and Pomonis 1995). This similarity in physical property suggest a distinct role for these methyl ethers in species recognition, as opposed to a more general physical role such as waterproofing. As stated earlier, methyl ethers are abundant and species-specific in some linyphiids and in *Nephila clavipes* and have been proposed to play a role in species recognition in spiders (Schulz 2013). It is likely that these compounds act as contact, rather than olfactory, signals since they are large molecules of low volatility. However, as our bioassays involved contact with silk, we cannot separate olfactory from gustatory detection. Their identification enables us, in future studies, to design syntheses and tests of biological activity, in both contact and olfactory assays.

Interestingly, extracts of *Tetragnatha* males and females did not differ. In spiders, pheromones for attracting maties are generally produced by only one sex, usually females (Chinta et al. 2010; Schulz 2013; Schulz and Toft 1993a), although there is one case in which males produce the pheromone (Xiao et al. 2010). That both sexes of each of the *Tetragnatha* species produce the same methyl ethers suggests that the composition of the mixture may be important. It is also possible that other compounds (e.g., more volatile or more polar), not detected by our analytical approach, might also serve as sex recognition cues, and might differ between the sexes. Future studies will investigate headspace methods and other solvents for sampling volatile compounds and chemicals not readily soluble in DCM.

We found no differences in extracts between virgin and mated females. This was a little surprising in light of studies on other species that have shown a rapid decline in signaling in mated females (Schulz 2013; Uhl 2013). The selective advantage for this decline may be that females benefit from switching off pheromone production or that males manipulate females via accessory secretions that are transferred with the sperm to prevent remating with another male (Herberstein et al. 2011). That *Tetragnatha* females continue producing methyl ethers could support a role in continuing species recognition (e.g., avoiding heterospecifics). However, it could also suggest that females benefit from mating with several males (Danielson-François et al. 2002; Danielson-François and Bukowski 2005).

Conclusion

Our results show that *T. extensa* males use chemicals to distinguish conspecific from heterospecific females. The chemical profiles of these spiders differ in the number and ratio of different methyl ethers, suggesting that these compounds may be responsible for species recognition in *Tetragnatha* and possibly other spider species in which these compounds have been found (Schulz 2013). Identification of these compounds paves the way for their synthesis and testing of biological activity.

Figures & Tables

Fig. 1 – (**A**) Silk choice trial and (**B**) silk extract choice trial setups (not drawn to scale). Males were released at the far end of the center stick.

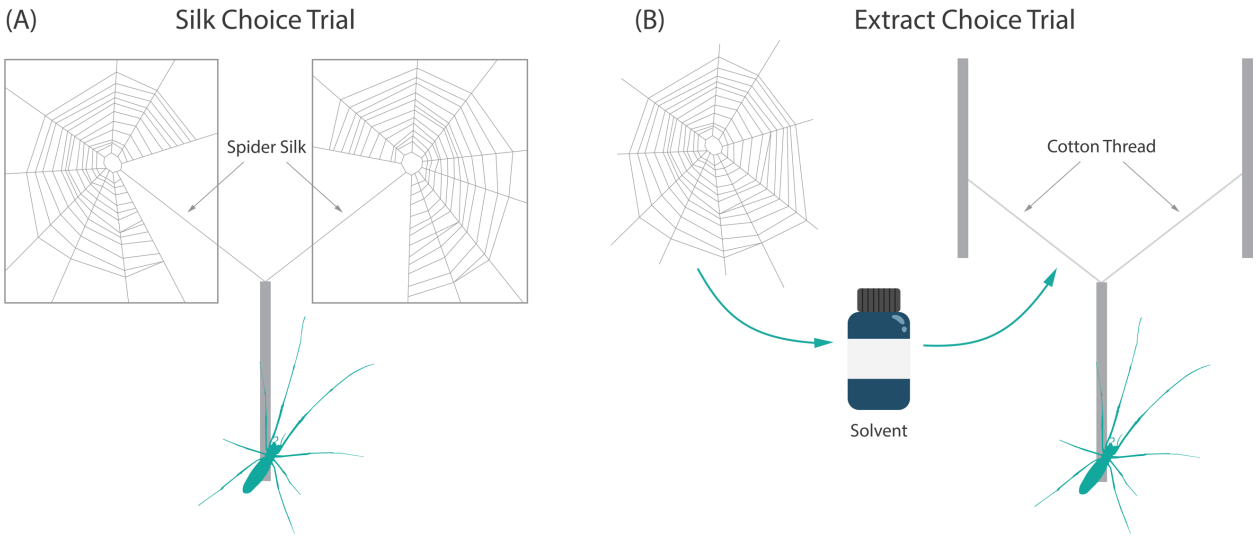


Fig. 2 – Results of male *Tetragnatha* extensa choice trials in which spiders were given a choice between conspecific and heterospecific female silk (Silk, Chi-square test, $P = 0.005$), and between extract of conspecific and heterospecific female silk applied on a cotton thread (Extract, Chi-square test, $P = 0.005$).

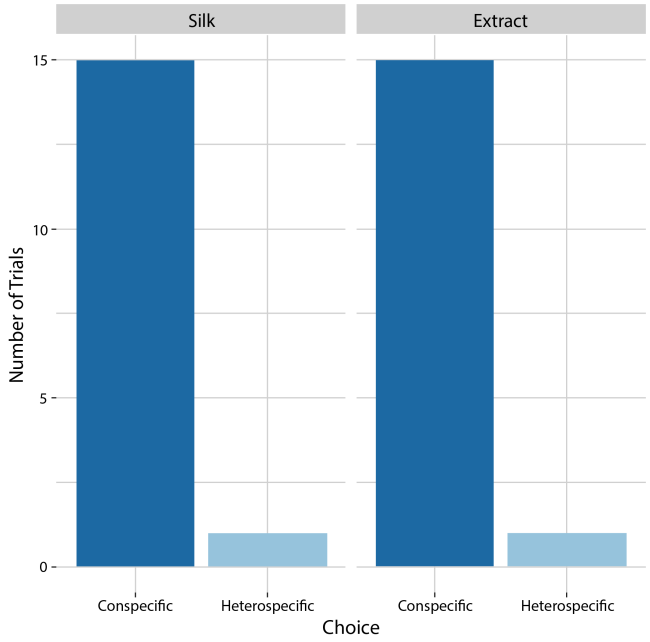


Fig. 3 – Total ion chromatograms (TICs) of body extracts of *Tetragnatha extensa* (upper) and *T. versicolor* (lower). Methyl ethers are indicated as compounds **A**-**D**. The similar TICs of other sample types are given in the supplementary material.

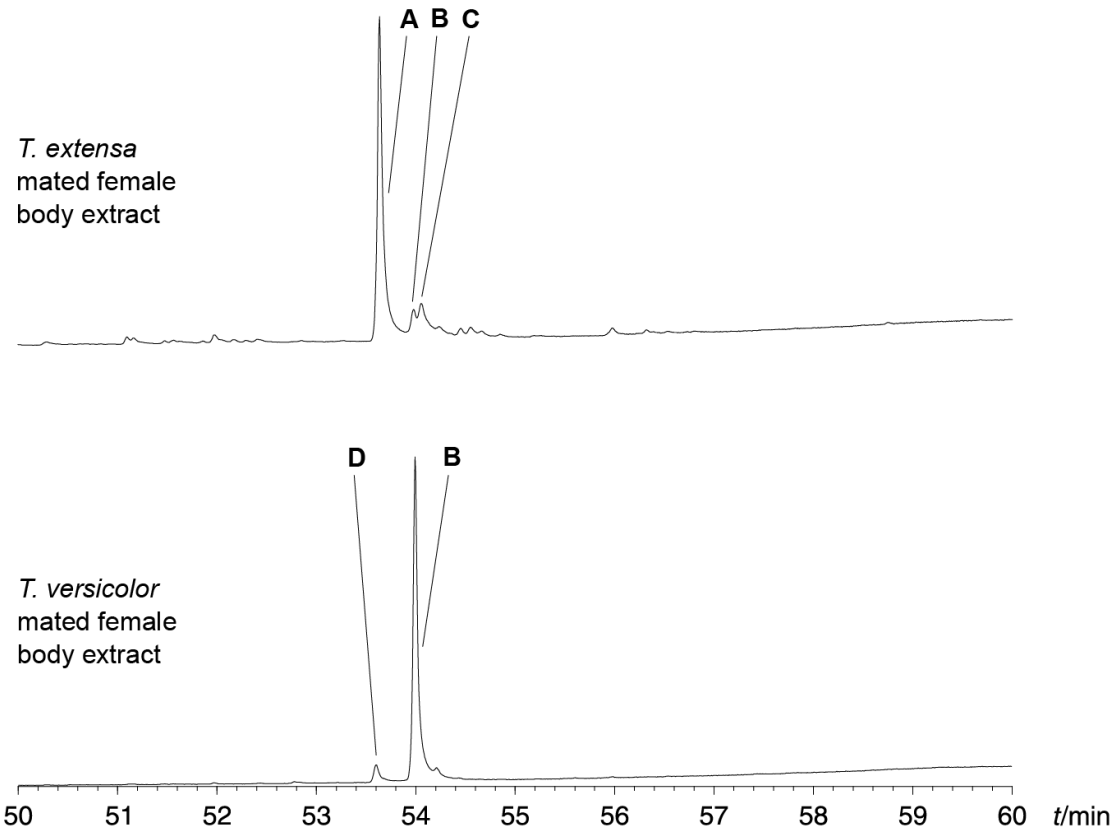


Fig. 4 – Mass spectra of compounds **A**-**D** present in body extracts of *Tetragnatha extensa* (**A**, **B**, **C**) and *T. versicolor* (**B**, **D**). The ion at *m/z* 45 is characteristic for methyl ethers.

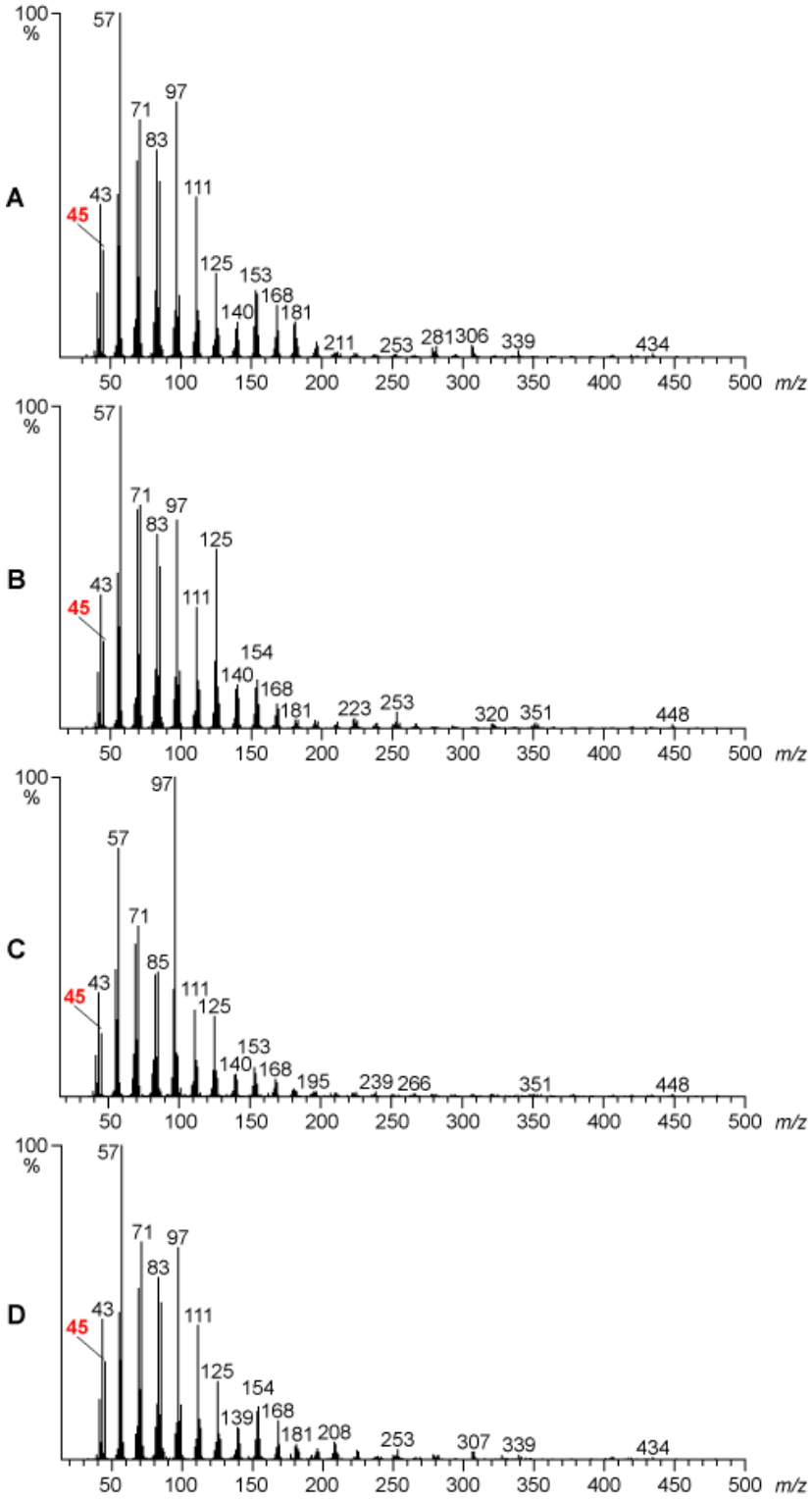
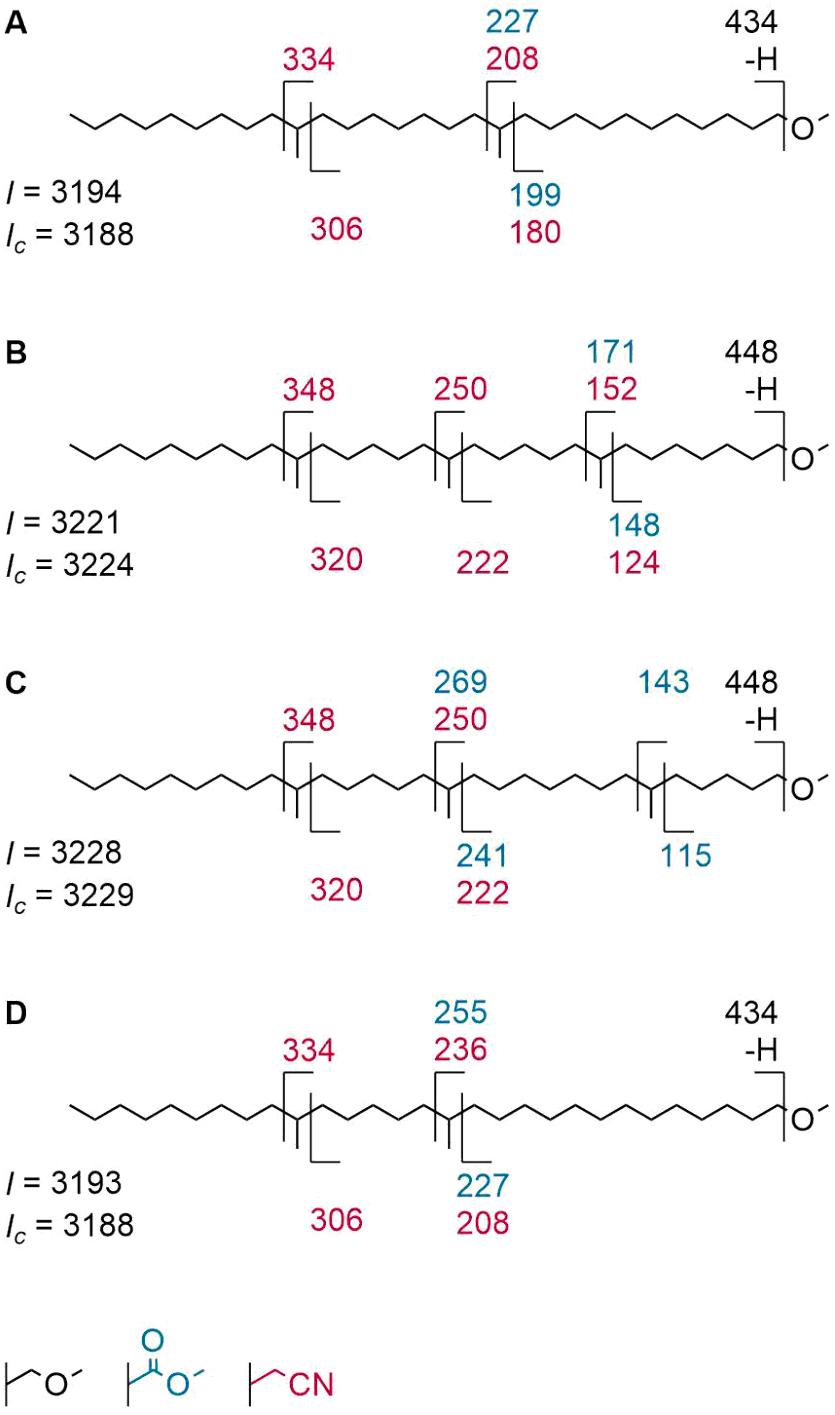


Fig. 5 – Proposed structure of compounds **A**, 12,20-dimethylnonacosyl methyl ether, **B**, 8,14,20-trimethylnonacosyl methyl ether, **C**, 6,14,20-trimethylnonacosyl methyl ether, and **D**, 14,20-dimethylnonacosyl methyl ether. Calculated (I_c) and experimental (I) gas chromatographic retention indices, and characteristic ions in the various mass spectra of the compounds **A-D** (black) and methyl ester (green) and nitrile (red) derivatives, indicating the location of methyl branches (for spectra see supplementary material).



Supplemental Material

Figure S1: Total ion chromatogram (TIC) of web and body extracts of female *Tetragnatha extensa*.

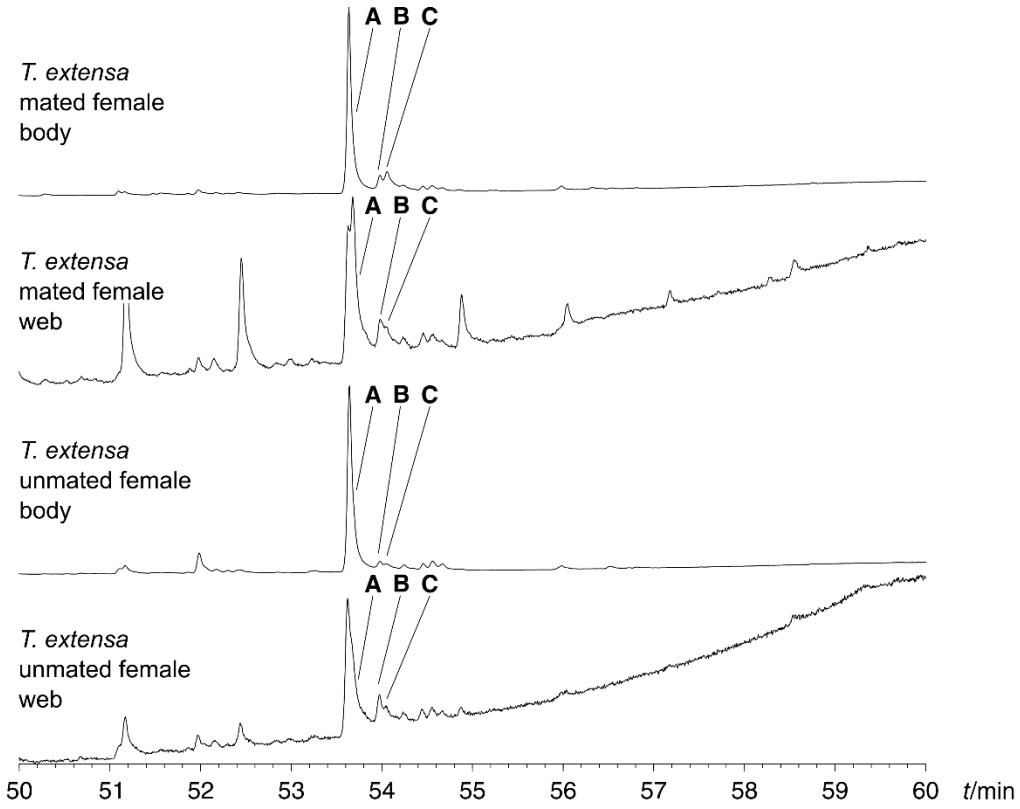


Figure S2: TIC of body extracts of male *Tetragnatha extensa*.

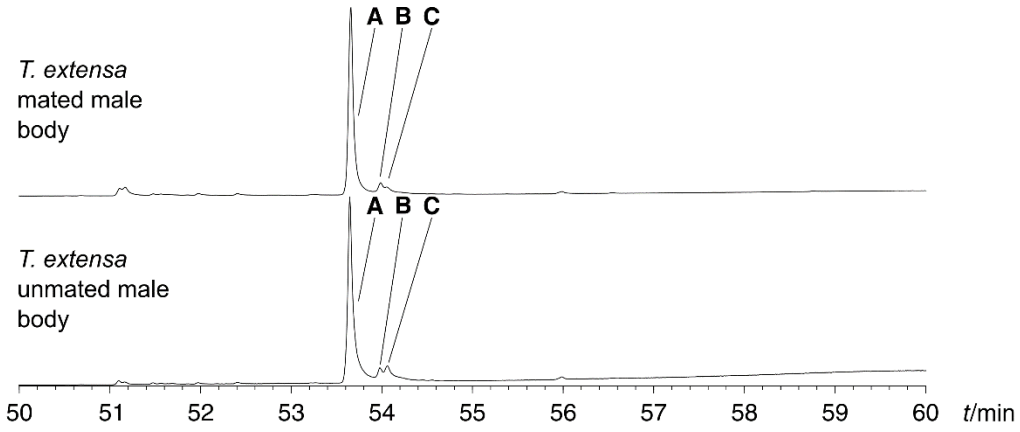


Figure S3: TIC of silk and body extracts of female *Tetragnatha versicolor*.

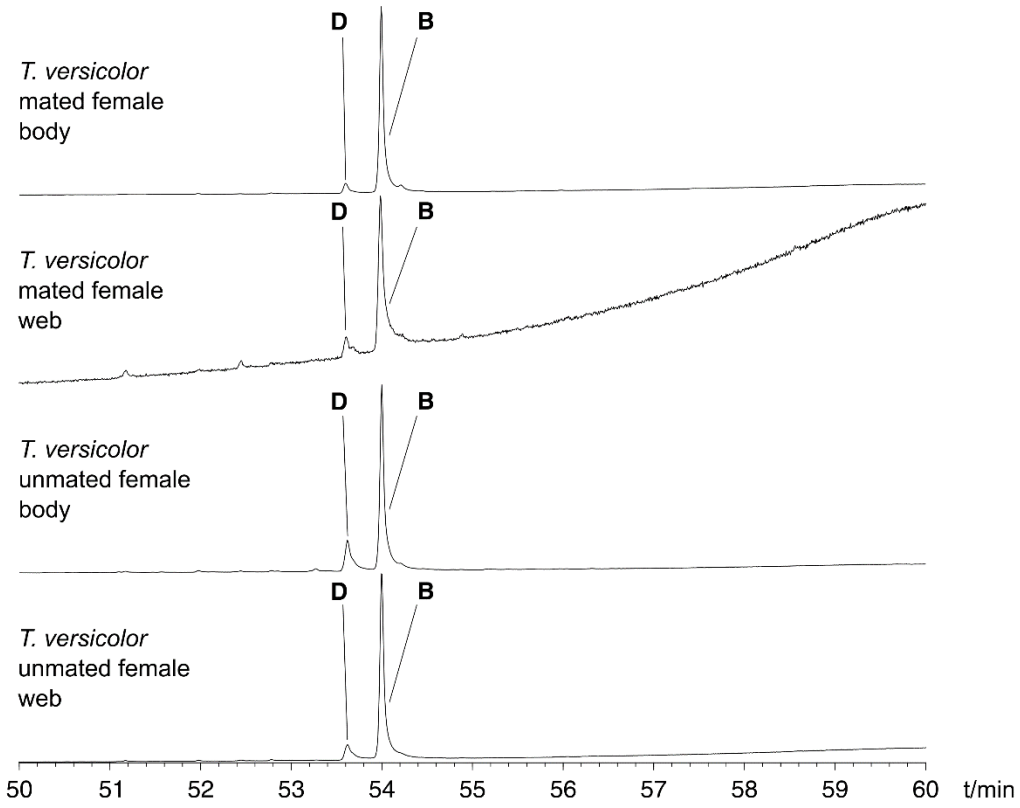


Figure S4: TIC of body extracts of male *Tetragnatha versicolor*.

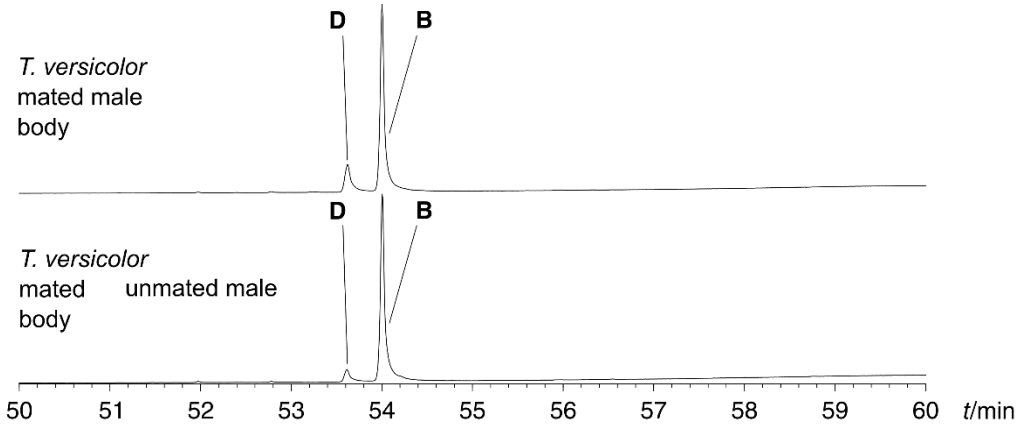


Figure S5: Mass spectra of methyl ethers **A**-**D**.

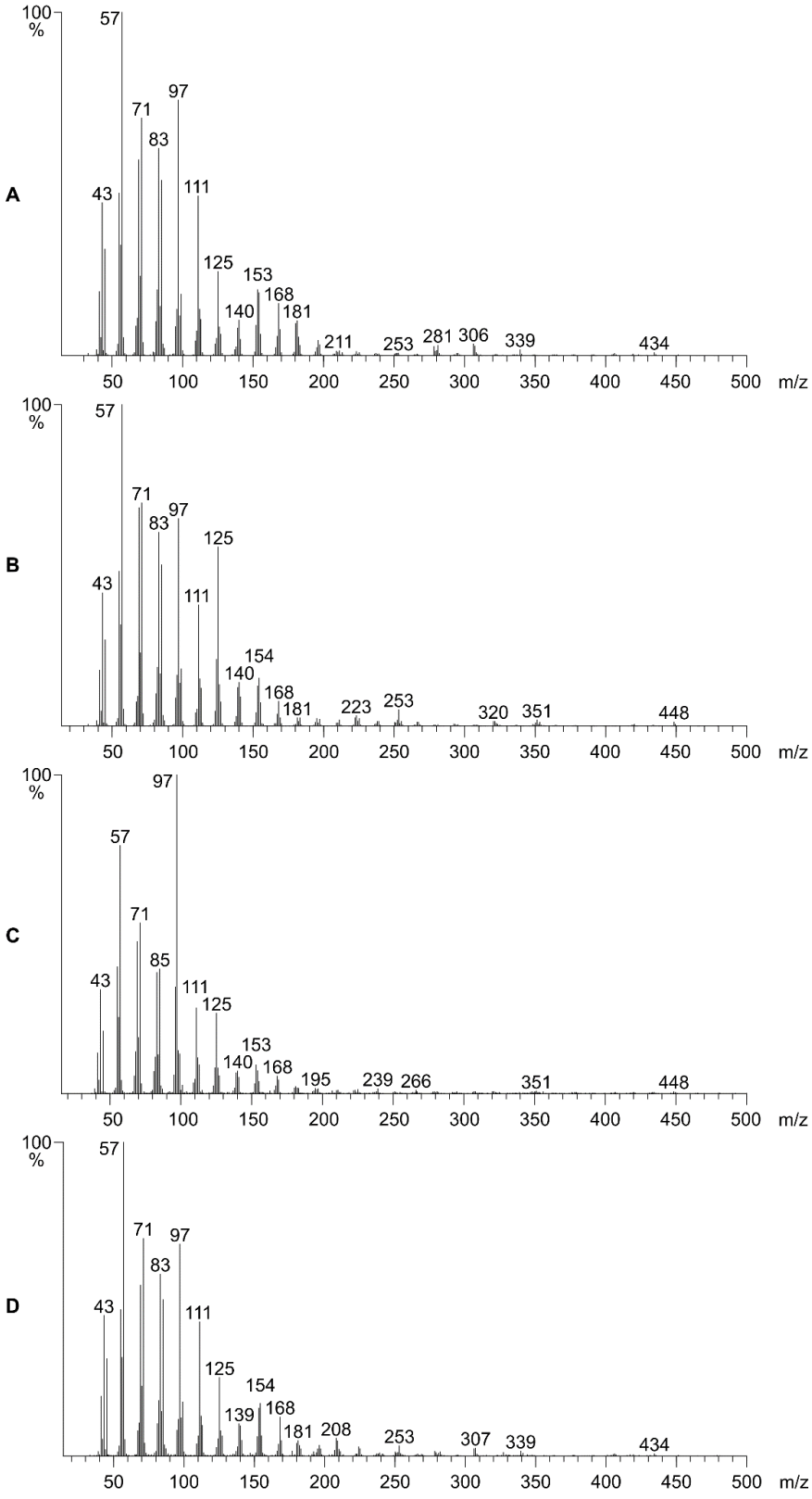


Figure S6: Mass spectra of methyl esters **A**-**D** derived from the respective methyl ethers.

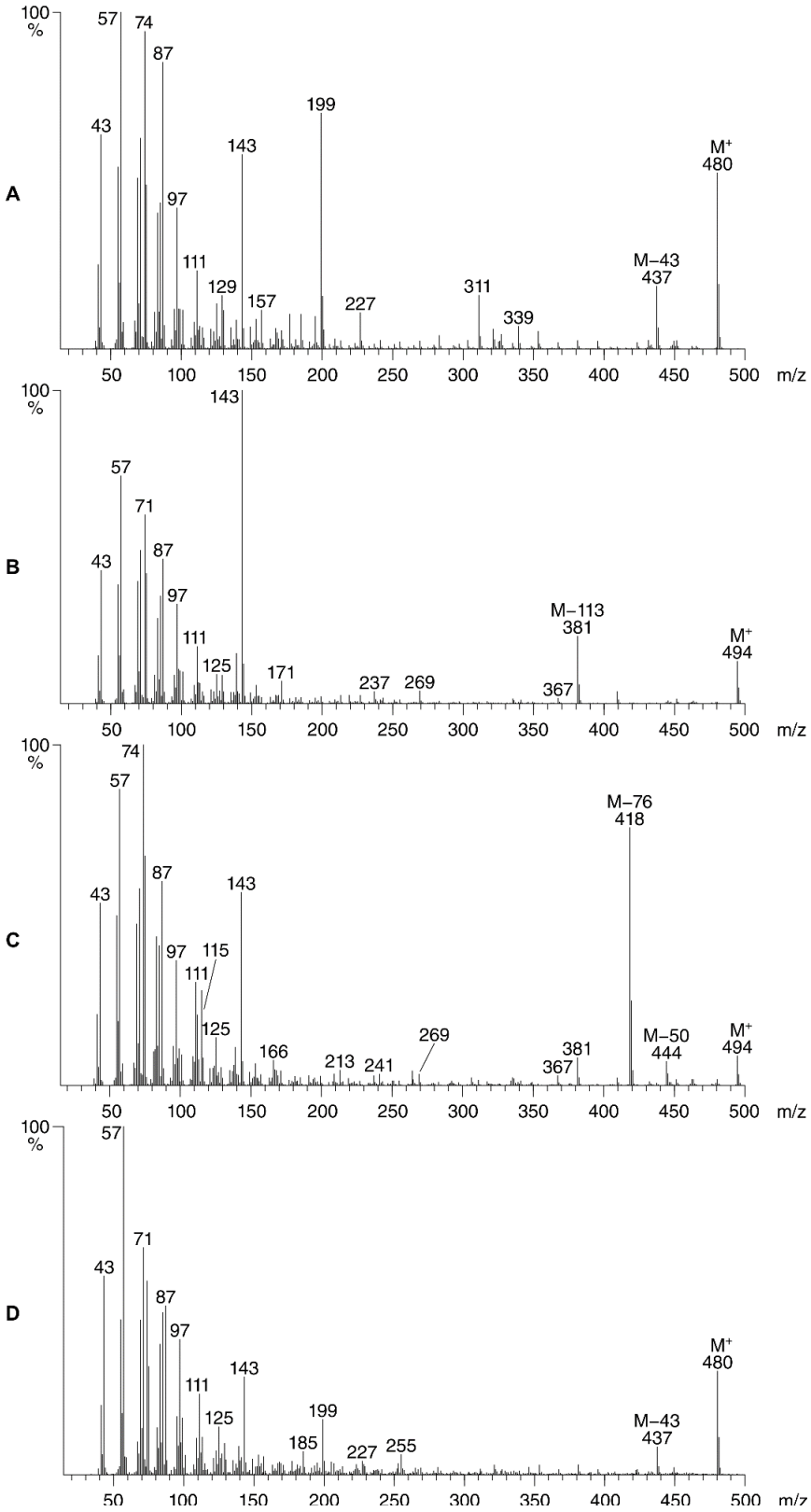
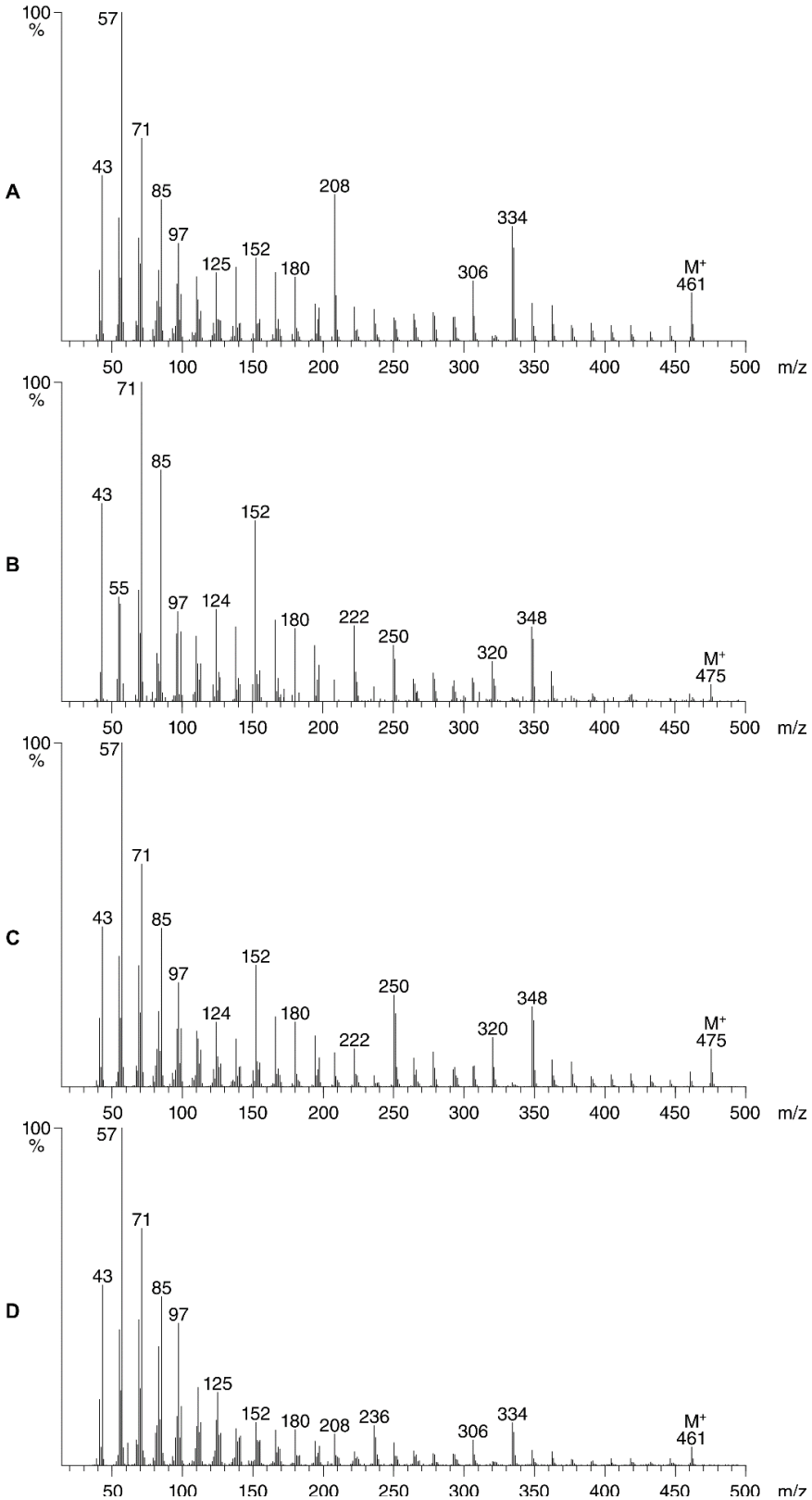


Figure S7: Mass spectra of nitriles **A**-**D** derived from the respective methyl ethers.



Acknowledgments

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In the next chapter, I take the established protocols from Chapter 1 and apply it to study the chemical species recognition cues of the closely related co-occurring species of Hawaiian *Tetragnatha* spiders that have adaptively radiated across the Hawaiian archipelago.

Chapter 2

Chemical Species Recognition in an Adaptive Radiation of Hawaiian *Tetragnatha* Spiders (Araneae: Tetragnathidae)

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In revision for Current Biology

Abstract

The mechanisms by which rapid reproductive isolation is attained and the relative role sexual signals play in the process has received much discussion. Yet it is still unclear how reproductive isolation can be attained so rapidly especially in systems with no obvious visual nor auditory signals at play. In this study we explore the use of chemical signals in the species recognition and adaptive radiation of Hawaiian *Tetragnatha* spiders by conducting behavioral assays and analyzing the chemicals found on their silks using gas chromatography-mass spectrometry. Male spiders significantly preferred compounds of conspecific mates, long chain alkyl methyl ethers. These lipids were remarkably species-specific in the combination of various methyl ethers. The differences were greatest between co-occurring species and between closely related sister species. These findings provide key insights into the role chemical cues play in the rapid divergence of species and the maintenance of reproductive barriers between recently diverged species.

Introduction

A common feature within adaptive radiation is the co-occurrence of multiple species that are ecologically well differentiated, yet very closely related. This situation begs the question of whether, and if so how, reproductive isolation is achieved. On the one hand, sexual recognition may drive evolutionary change (Broder et al. 2021); on the other, it may be a by-product of adaptation to different niches or environments (Gilbert and Bell 2018). Identifying the role of sexual recognition is fundamental to understanding the interplay between sexual selection, reinforcement, ecological selection, and character displacement, in speciation, which in turn affects patterns of species accumulation, niche shifts, and co-occurrence (Gillespie et al. 2020). The current study set out to examine the possible role of sexual selection and reinforcement within an adaptive radiation of organisms in which it has been assumed that speciation is driven by niche-based divergence (Gillespie 2004; Blackledge and Gillespie 2004).

An early argument for rapid species formation during adaptive radiation focused on divergence in reproductive attributes due to the small sample size of the new population (Ernst 1954). The stochastic shifts in the genetic environment may lead to reproductively isolated taxa being formed almost instantly (Ernst 1954; Templeton 2008; Sendell-Price et al. 2021). Such founder effects can also lead to shifts in the behavioral repertoire among founding populations, with this mechanism being proposed to drive divergence in the behavioral repertoire of the large radiation of picture-winged Hawaiian *Drosophila*, each species with its own set of visual and vibratory signals (Kaneshiro 1980, 1983; Templeton 2008). Similarly, within the radiation of Hawaiian crickets in the genus *Laupala*, changes in the signaling environment due to founder effects during colonization is proposed to have caused the rapid change in visual and auditory cues, leading females to discriminate among divergent songs, with correlated evolution of song and preference potentially acting as a driver of diversification in the radiation (Oh, Conte, and Shaw 2013; Shaw and Lugo 2001).

Alternatively, reproductive isolation in rapidly evolving adaptive radiation may be achieved as a by-product of adaptive evolution (Dobzhansky 1951). In cases where the same morphological traits that undergo adaptive change also function in mate recognition, so-called “magical traits” (Servedio et al. 2011), it is straightforward to envision the process (Dobzhansky 1951; Ernst 1963). Such a mechanism has been implicated in the changes to beak morphology that are associated with both the diet and mating song in Galapagos finches (Podos 2001), as well as changes in body coloration in Stickleback and Cichlid fish populations that are associated with shifts in both the feeding niche and the signaling light environment (Boughman 2001; Maan et al. 2006; Seehausen et al. 2008; Maan, Seehausen, and Groothuis 2017). However, in many other taxa, close relatives can co-occur without evident differences in behavioral or morphological repertoires that can be linked to reproductive isolation between species. Thus, the age-old question is whether adaptive, ecological factors alone can provide a barrier that is sufficient enough to yield robust reproductive isolation. The current study focuses on an adaptive radiation of spiders where multiple species co-occur with marked niche differences within a site, yet males are often found wandering between niches looking for females (Kennedy et al. 2019). Despite this, there is little evidence of behavioral differences in courtship (Gillespie 1991) and hybridization between co-occurring species is uncommon (Cotoras et al. 2018). Our question was whether species may be using an alternative sensory modality, notably that of chemistry, and

if so, whether the signals were sufficiently divergent to distinguish between co-occurring taxa at a site.

The Hawaiian Islands are particularly well known for many examples of adaptive radiation. Among spiders, the larger radiations are nocturnal. The genus *Tetragnatha* in particular comprises over 50 different species across the archipelago. While generally known outside Hawaii for its uniform brown coloration, long legs and long abdomen, and a propensity to build horizontal orb webs over water (Levi 1981), in Hawaii they exhibit a wide array of morphologies and ecologies. Overall, the lineage is divided into two clades - the "web-building" clade that builds orb-shaped webs in the vegetation, and the "spiny leg" clade that have abandoned web building and live a cursorial lifestyle (Gillespie 1991, 1992, 2004; Blackledge and Gillespie 2004). Representatives from these two clades can be found on every island. However, like their mainland counterparts, they have low visual ability and show little evidence of vibratory communication, even among the web-builders which generally have flimsy webs (Levi 1981) with the strands often water-drenched in Hawaiian rainforest taxa (Gillespie 1992) making them seemingly unfit to transmit vibratory cues; moreover, the spiders lack stridulatory structures (Danielson-François, Fetterer, and Smallwood 2002; Levi 1981; Gillespie 1991, 1992). Yet, despite the lack of obvious signaling modalities, and the frequent co-occurrence of multiple species, eight or more, at a site (Gillespie 1991, 1992), there is little evidence of hybridization (Cotoras et al. 2018) and populations of sister taxa are almost invariably highly structured (Cotoras et al. 2018; Roderick et al. 2012). The lack of evidence in the use of either visual or vibrational cues in courtship led to the assumption that the strong reproductive isolation observed between sister taxa of some of the major lineages was driven almost entirely by ecological differences and natural selection (Gillespie 2004; Blackledge and Gillespie 2004). However, one major signaling modality – that of chemicals – has not yet been examined.

Although relatively unknown compared to visual and auditory cues, it is now clear that shifts in chemical cues can also play a role in facilitating divergence between populations, and hence speciation (Smadja and Butlin 2009; Adams and Tsutsui 2020). Species-specific chemical differences are particularly well known in insects where chemical cues have contributed to both the creation and reinforcement of reproductive isolation between insect species by means of natural selection to desiccation resistance (Chung and Carroll 2015), habitat choice (Caillaud and Via 2000; Linn et al. 2003), pollinator choice (Brand et al. 2020), and reproductive character displacement (Higgie, Chenoweth, and Blows 2000; Dyer et al. 2014; McElfresh and Millar 2001). Chemical cues involved in reproductive isolation can evolve rapidly, creating new compounds or changing the relative amount of a suite of compounds with sometimes just the change of a single gene or codon (Smadja and Butlin 2009; Adams and Tsutsui 2020). When close relatives co-occur, as is common in adaptive radiations, chemical cues are seen to also play a role in the reproductive isolation of closely related taxa.

The current study set out to examine the role of chemical cues as a means for recognition among close relatives of Hawaiian *Tetragnatha*. Chemical cues have been well documented as playing an important role in species divergence across many other taxa such as insects, fish, mice and lizards (Smadja and Butlin 2009; Adams and Tsutsui 2020) and have the potential to allow recognition between closely related and co-occurring species within a radiation. Previous work on two continental species of *Tetragnatha* spiders showed that male *Tetragnatha* can distinguish

conspecific from heterospecific mates using long chain alkyl methyl ether lipids found on the silk of female webs (Adams et al. 2021) . From this we can hypothesize that these methyl ethers found on the silk of female spiders may play a role in species recognition and hence act as a reproductive barrier between co-occurring species of Hawaiian *Tetragnatha*. We focus on two sites within the island of Maui to investigate the role of chemical cues in the species recognition of sympatric and allopatric Hawaiian *Tetragnatha* spiders and determine the extent to which any signal might be species-specific, how the compounds in the signal have evolved structurally, and congruently, whether the signal divergence could be a product of the chemical diversity within a given site. Specifically, focusing on methyl ethers, which have been proposed to serve as species recognition cues in *Linyphia* spiders (Stefan Schulz 2013), we ask: (1) whether these chemicals derived from the silk allows species to separate conspecifics from heterospecifics. We then test (2) whether co-occurring species have methyl ethers that are entirely structurally unique from one another or differ in abundance as seen in many insects, while there may be overlap between species that do not co-occur. Finally (3), if the chemical identity of the set of species that make up the community of co-occurring taxa is driving signal divergence, then we expect to see chemical differences between populations of the same species so as to reflect the chemical signaling environment of the local community in which that population occurs. We find that male Hawaiian *Tetragnatha* spiders use chemicals found on the silk of females to distinguish conspecifics from heterospecifics, and that these chemicals are extremely species-specific with little to no overlap found in the specific methyl ethers used between closely related co-occurring species. The chemical profile of the closely related species differed not only in the amount of a given methyl ether, but also in the actual structure of these chemicals. Our results show the key role of chemical cues in providing a vehicle for reproductive isolation in the course of adaptive radiation.

Materials & Methods

Study Organisms

Adult female, adult male, and juvenile spiders of 11 different Hawaiian *Tetragnatha* species were collected from The Nature Conservancy of Hawaii's Waikamoi Preserve on the Hawaiian island of Maui during the summers of 2016-2020. The majority of spiders were collected from two sites – Upper Waikamoi at 1800 – 1950 m and Lower Waikamoi at 1380 – 1500 m. At Upper Waikamoi, *T. eurychasma*, *T. kamakou*, *T. quasimodo, T. stelarobusta, T. trituberculata,* and *T. waikamoi* can be found in sympatry while at Lower Waikamoi *T. brevignatha, T. filiciphilia*, *T. quasimodo, T. restricta, T. stelarobusta,* and *T. waikamoi* can be found in sympatry (Gillespie 1992). On the other hand, *T. acuta* was collected at a higher elevation site just above Upper Waikamoi at around 2000 m, while the undescribed "Crater Elongate Forest", a dry habitat specialist, was collected along the Haleakala Supply Trail and inside Haleakala Crater at 2000 – 2200 m. California *Tetragnatha* spiders, *T. pallescens* and *T. versicolor* were collected from Bodega Marine Reserve and Angelo Coast Range Reserve respectively. Once collected, specimens were housed in modified 11 x 7 x 6 cm Tupperware containers with wet cotton balls for moisture and a plastic stick for hiding. The Hawaiian spiders were then placed in a large incubator set to a thermal cycle of 10°C to 15°C and a 12hr light and 12hr dark cycle while the

California spiders were housed at room temperature with a 12hr light and 12hr dark cycle. All spiders were fed once a week with roughly 4 *Drosophila hydii* or 8 *Drosophila melanogaster* each.

Silk & Silk Extract Choice Trials

To assess whether males of Hawaiian *Tetragnatha* spiders could discriminate conspecific from heterospecific females using silk produced by the females, dragline (major ampullate) silk strands of conspecific and heterospecific females were presented to male spiders in a silk choice trial (Figure 1a). We used the same protocol as that described in our recent work (Adams et al. 2021). Conspecific and heterospecific females were individually taken out of their Tupperware containers and manipulated with two bamboo sticks so that the spider naturally produced dragline silk as they walked across the sticks. The dragline was then carefully placed so that one end of the silk strand was attached to one of the two vertical sticks and the other end was attached to the horizontal stick centered between the two vertical sticks. The same procedure was repeated with the dragline from the other female (either conspecific or heterospecific) using new, clean bamboo sticks until two silk strands were attached to the horizontal stick in a Y-formation at a slight upward angle of about 20°. All tools were washed with 70% ethanol, soap, and water and dried overnight after every trial. A male was then gently placed on the opposite end of the horizontal bamboo stick facing the silk strands. If the male did not move from this position within 30min after placing him onto the stick, the male was gently removed from the stick and replaced with a new male. A new, cleaned bamboo stick was used only if the male moved from the starting position up the stick thereby potentially leaving a chemical or silk trail on the stick. The males that moved within 30min after placing them on the stick all walked up the stick toward the silk strands and came in contact with the strands with their first and second pair of legs. The male was considered to have made a choice when he moved off the stick and walked up one of the silk strands. All trials were conducted in the dark under a dim red light to mimic nocturnal conditions and recorded using a smartphone camera. The videos were then checked to see if the males touched both silk strands since direct contact with the silk was considered necessary to perceive contact chemical cues. A total of 17 trials were conducted with *T. brevignatha*, 21 trials with *T. eurychasma,* 17 trials with *T. quasimodo,* 20 trials with *T. stelarobusta,* 14 trials with *T. trituberculata,* and 16 trials with *T. waikamoi* males. For each species tested, conspecific females from the same site and co-occurring heterospecific females from the same site were chosen (Specific combinations listed in SI 19). Heterospecific species from the same clade (spiny leg clade or web building clade) were chosen for all species except for *T. brevignatha* and *T. stelarobusta*.

To assess whether males of Hawaiian *Tetragnatha* spiders could discriminate conspecific from heterospecific females using chemical cues alone, chemicals from the dragline silk of conspecific and heterospecific females were extracted with dichloromethane (DCM, analytical grade) and the extracts were presented to males on cotton threads in a silk extract choice trial (Figure 1b). To make the silk extracts, dragline silk naturally deposited inside the Tupperware homes were collected by wrapping the silk around a clean metal spatula and then pushing it into a tight ball using clean tweezers. The silk ball was then placed in a 2mL glass vial (Agilent Technologies) and soaked in 0.2mL of DCM for 30min. After 30min, clean tweezers were used to remove the silk ball, the vials were closed and secured with parafilm, and the extracts stored at -20 °C until

further use. Behavioral trials conducted with these silk extracts were done according to the protocol described in our recent work (Adams et al. 2021). Two pieces of cotton thread 15cm in length were attached to each vertical and horizontal bamboo stick using painter's tape to form a Y-shape. The cotton threads were also sloped upward at an angle of about 20°. Silk extracts of conspecific and heterospecific females were dispensed with a clean glass pipette onto each cotton thread. The application of conspecific and heterospecific extract were alternated for each trial between the two cotton threads. Once applied, the extracts were allowed to set for 1min, giving time for the DCM to evaporate. A male was gently placed at the opposite end of the bamboo stick in the same manner as in the silk choice trials. The male was considered to have made a choice when he moved off the bamboo stick and walked up one of the cotton threads. All trials were conducted in the dark under red light and recorded using a smartphone camera and again, only males that had touched both cotton threads were accounted for in the analysis. A total of 23 trials were conducted with *T. brevignatha*, 21 trials with *T. eurychasma,* 30 trials with *T. quasimodo,* 29 trials with *T. stelarobusta,* 17 trials with *T. trituberculata,* and 25 trials with *T. waikamoi* males. As with the silk choice trials, for each species tested in the silk extract choice trials, silk extracts of conspecific females from the same site and silk extracts of co-occurring heterospecific females from the same site were chosen (SI 17).

Chemical Analysis of Spider Extracts

Dragline silk extracts of *Tetragnatha* spiders were analyzed by GC-MS (Agilent 7890A/5975C). Dragline extracts from 22 *T. acuta,* 29 *T. brevignatha,* 7 *T. eurychasma*, 23 *T. filiciphilia*, 11 *T. kamakou*, 7 *T. pallescens,* 33 *T. quasimodo,* 8 *T. restricta,* 28 *T. stelarobusta,* 21 *T. trituberculata,* 18 *T. versicolor,* 30 *T. waikamoi* and 5 "Crater Elongate Forest" were analyzed. The gas chromatography was fitted with a fused silica capillary column (Agilent, DB-5MS, 30 m x 0.32 mm x 0.25 μm) with helium as the carrier gas. Extracts were analyzed in splitless mode, with a column oven temperature program of 50 °C for 5 min, increased by 5 °C min^{-1} to 320 °C. Injector and transfer line temperatures were maintained at 250 °C.

GC-MS data was analyzed in OpenChrom (Wenig and Odermatt 2010) by detecting chromatogram peaks using the First Derivative Peak Detector feature at the highest threshold. Peak integration areas and linear retention indices were calculated using the Trapezoid Peak Integrator and Retention Index Calculator features respectively. Previous work showed that methyl ethers are the most abundant compound found in the *Tetragnatha* spider silk extracts and were species-specific (Adams et al. 2021). As such, in this study, peaks with a strong *m/z* 45 were identified as methyl ethers (Stefan Schulz and Toft 1993) and added to a user-built methyl ether library. Methyl ether peaks were aligned manually, based on retention time, retention index, and visual comparison with spectra in the library. For each individual chromatogram, relative peak contributions of each methyl ether were calculated by dividing the peak area of a specific methyl ether by the sum of the peak areas of all methyl ethers found in the sample.

Structural Analysis of Compounds

To assess the structural evolution of the methyl ethers found across the spiders, the structures of the most common methyl ethers found in each species were determined by the derivatization of the methyl ethers in two separate reactions using previously established procedures (Adams et al.

2021): transformation into cyanides via iodination (Stefan Schulz 1997) and separate oxidation with RuO_4 into methyl esters (S. Schulz 2001). In the iodation cyanation reactions, conversion of methyl ethers to corresponding nitriles was done in two steps. First, the methyl ethers were converted to iodides through the addition of trimethylsilyl iodide (4 µl) to the natural extract (10 µl), heating at 60 °C for 2h and shaking intermittently. The reaction was stopped by adding saturated sodium bicarbonate solution (40 µl), and the mixture was extracted with DCM (3 x 40 µl). The organic phase was separated from the aqueous phase and collected in a 1.5 µl GC-MS vial. Sodium chloride (3 mg) was used to dry the mixture. The solution was extracted using a pipette, and the remaining sodium chloride was washed with DCM (3 x 400µl) to capture any remaining solution. The combined solutions were placed into a new 1.5 ml GC-MS vial, and tetraethylammonium cyanide (1 mg) was added. The reaction mixture was stirred for 16 h at room temperature. The reaction was stopped through the addition of water (200 µl) and vigorous stirring for 10 minutes. The aqueous phase was then removed using a pipette. The remaining sample was dried to remove the solvent under a stream of nitrogen and triturated with pentane (3 x 100 µl). The residue was then redissolved in DCM (10 µl) for use in GC/MS analysis. In the oxidation reaction, methyl ethers contained in the natural extracts were converted to their corresponding methyl ester derivatives using ruthenium tetroxide as an oxidizing agent. A sample was initially prepared by adding natural extract (10 µl), CCl_4 (5 µl), RNase-free water (75 µl), MeCN (50 µl), and sodium periodate (10 mg) to a 1.5 GC-MS vial. Ruthenium (III) chloride (1 mg) was added to the vial, and the mixture was stirred vigorously at room temperature for 2 hours. The golden organic phase was separated from the black aqueous phase with DCM (3 x 200 µl), combined, further diluted with diethyl ether (500 µl), and dried by elution through a small pipette filled with sodium sulfate. A new pipette containing silica gel was then washed with diethyl ether (3 x 200 µl) and the combined dried mixture was filtered through to obtain the final sample. The solvent was then evaporated using a nitrogen stream and redissolved in DCM (10 µl) for GC-MS analysis.

Using the information from the derivatives, we were able to determine the total carbon number including the methoxy group, the methyl branching pattern, the ω-branching pattern represented as the methyl branching pattern counted from the methoxy group, the carbon backbone length, and the elongation amount represented as the number of carbons between the methoxy group and the methyl branch closest to the methoxy group. ω-branching patterns were examined in this study since these methyl ethers are most likely derived from fatty acid biosynthesis and are proposed to be constructed from the end opposite to the methoxy group, similar to insect hydrocarbons (Chinta et al. 2016; Millar 2010). Average values for each structural category were then calculated for every species by averaging the structural properties of the methyl ethers found in all the individual profiles of a given species.

Statistical Analysis – Chemical Analysis of Spider Extracts

To assess whether the chemical profiles of Hawaiian *Tetragnatha* exhibit species-specific patterns both in the identity and abundance of methyl ethers, a non-metric multidimensional scaling (NMDS) ordination plot was created using the individual-level dataset in the vegan package in R run with 2 dimensions and 100 iterations (Oksanen et al. 2019; Team 2020). Shepard plots were created to see goodness of fit. To test for significant differences in chemical profiles between species, Permutational Multivariate Analysis Of Variance Using Distance

Matrices (PERMANOVA) was performed using the adonis function in the vegan package (Oksanen et al. 2019). Since PERMANOVA is sensitive to differences in intra-group dispersions (Anderson 2001), an analysis of Multivariate Homogeneity Of Groups Dispersions (PERMDISP) was performed using the betadisper and permutest functions, also in the package vegan. All methyl ether profile dissimilarities were calculated using the Bray-Curtis index. Significance was assessed via 999 permutations. To assess differences in chemical profiles between sympatric species, a pairwise PERMANOVA (Hervé and Hervé 2020) was performed for sympatric species pairs. The Benjamini-Hochberg method was used to adjust the p-values. A Tukey post-hoc test was performed on the group dispersions (calculated with betadisper) to determine differences between sympatric pairs. Lastly, to assess whether there were differences in the chemical profiles between the sexes and populations within a species, PERMANOVA and PERMDISP analyses were also performed on species grouped by sex – male vs female in *T. acuta, T. brevignatha, T. filiciphilia, T. kamakou, T. quasimodo, T. stelarobusta, T. trituberculata,* and *T. waikamoi*, and population location – Upper vs Lower Waikamoi in *T. stelarobusta*.

To incorporate phylogenetic data into our visualizations of NMDS data, a reduced phylogenetic tree based off the tree from Kennedy et all 2021 (Kennedy et al., n.d.) was projected onto a two-dimensional chemical space defined by our NMDS plot. The position of the end nodes in chemical space were calculated by taking the centroid of the points plotted on the NMDS for each species, and phylogenetic relationships were depicted through solid black lines extending vertically through time. The end points were also colored according to the phylogenetic branch length to the nearest node corresponding to each species. This was accomplished using the ChronoPTS2D function in the "evoldiver" package (Sakamoto 2022) combined with the package "rgl" (Murdoch and Adler 2021) for generation of a rotating 3-dimensional gif.

A table of the most common methyl ethers were made for each species by listing methyl ethers that were found in more than 50% of the individuals in each species, and those that had an average relative abundance greater than 50%. This ensured that we captured compounds that were found in large amounts as well as those compounds that were found in lower amounts but found consistently between individuals of a given species. For species with significant difference between male and female methyl ether profiles (as determined by ADONIS), a shortlist of methyl ethers for each sex was determined, applying the same criteria. The two lists were then combined to give a species-level shortlist.

We were interested in comparing chemical distance between species pairs based on the most common methyl ether profiles for each species. Since most species pairs did not share any compounds, the commonly used Jaccard distance was unsuitable as it yielded 1 as the distance for 58 out of 78 possible species pairs. Similar analyses on organisms with few shared compounds computed distance as the number of differences in pheromone profiles. Using this distance measure, a t-test was performed comparing the number of unshared compounds between co-occurring and non-co-occurring species pairs.

Statistical Analysis – Structural Analysis of Compounds

To analyze the similarities in the structural patterns of the most common methyl ethers found across the species, maximum common substructure (MCS) was used as a metric for chemical similarity (Cao, Jiang, and Girke 2008; Wang et al. 2013). MCS calculations were conducted on the entire structure of the methyl ethers as well as the structures without the methoxy functional group. The MCS and subsequently the Tanimoto coefficient (Cao, Jiang, and Girke 2008) were then computed for all pairwise combinations of the most common methyl ethers. To yield a dissimilarity value between chemical pairs, the Tanimoto Coefficient was subtracted from 1. A distance matrix was created using the chemical-pair dissimilarities. All MCS calculations were performed using the R package "fmcsR" (Wang et al. 2013). Hierarchical clustering based on the distance matrix was used to construct a dendrogram and group the methyl ethers into clusters based on their structural similarity. The optimal number of clusters was determined by silhouette analysis. Cluster stability was assessed via bootstrapping with 1000 resamples using the function clusterboot in the R package "fpc" (Hennig and Imports 2020). Finally, ancestral state reconstruction of the odd and even ω-branching patterns found in the species was performed using the R toolkit "MBASR" (Heritage 2021) run for 10,000 generations and sampled every 100 generations.

Results

Male Spiders Choose Conspecific Females Using Silk & Silk Extracts

In all silk trials, Hawaiian *Tetragnatha* males chose to walk up the silk strand of the conspecific female silk with higher probability than the silk strand of the heterospecific female (Figure 1). This was also the case in the silk extract choice trials where Hawaiian *Tetragnatha* males chose to walk up the cotton thread with the conspecific silk extract with higher probability than the thread with the heterospecific silk extract (Figure 1).

Spiders Have Species-Specific Combinations of Methyl Ethers

A total of 124 unique methyl ethers were detected in the dragline silk extracts of the 11 Hawaiian *Tetragnatha* species and the 2 Californian *Tetragnatha* species analyzed (SI 1). Of these, a total of 32 methyl ethers were found to be the most abundant compounds found across the profiles of these 13 species, while 61 methyl ethers were found in only one or two samples and the rest found in low relative abundances across multiple samples (Figure 2; SI 1; SI 2). When individuals were grouped into the three categories of "Hawaiian web builders", "Hawaiian non-web builders", and "California web builders", the number of methyl ether compounds found in each group was significantly different in a one-way ANOVA ($F_{2,239} = 24.67$, $p = <0.001$) with Hawaiian web builders having significantly more methyl ether compounds in their extracts (Tukey post hoc test, 4.84 ± 3.66) than both Hawaiian non-web builders (2.15 ± 1.64, $p = <0.001$) and California web builders (3.24 ± 2.89, $p = 0.0304523$) (SI 3).

The NMDS results (Figure 3; SI 4) show that the methyl ether profiles of the individual spiders cluster strongly based on species, suggesting that the identities and amounts of methyl ethers found in the profiles are species-specific. There is very little overlap in the chemical profiles

across species but a few exceptions exist. *T. quasimodo* and *T. versicolor* show significant overlap due to 1-methoxy-8,14,20-trimethylnonacosane (ME 33_A), being a major contributor to both species' profiles. Similarly, *T. acuta, T. waikamoi, and T. filiciphilia* have overlapping methyl ether profiles with 1-methoxy-6,12,18-trimethylheptacosane (ME 31_A) shared across these species. This compound is the only major contributor to the methyl ether profiles of *T. waikamoi* and *T. filiciphilia,* leading to a near perfect overlap on the NMDS plot (Figure 3c). The clustering of chemical profiles based on species was supported by the Bray-Curtis distances between individual chemical profiles. The results show that an individual's chemical profile was more similar to those of individuals of the same species (conspecific Bray-Curtis distance, mean = 0.2878117, sd = 0.3770496) than to individuals of another species (heterospecific Bray-Curtis distance, mean = 0.8982817, sd = 0.2780292), and this difference was found to be significant (*df* = 3066.6, $p = < 0.001$). PERMANOVA analysis also supported a strong species effect on chemical profiles, suggesting significant difference between the species' chemical profiles (ADONIS: $F_{12,227} = 57.55$, $R^2 = 0.75262$, $p = 0.001$). Additionally, PERMDISP showed that different species had different levels of intra-species dispersion in chemical profiles (permutest: $F_{12,227} = 14.359$, $p = 0.001$). *T. acuta, T. brevignatha, T. eurychasma, T. stelarobusta* and *T. trituberculata* appear to have more disperse distributions of individual chemical profiles, while the rest appear to have less variance in chemical profiles between individuals (SI 5).

Between male and female specimens of the same species, PERMANOVA analyses showed that chemical profiles differed significantly between males and females in *T. acuta, T. brevignatha, T. stelarobusta,* and *T. trituberculata* but not in *T. filiciphilia, T. kamakou, T. quasimodo,* and *T. waikamoi*. (SI 6). The most remarkable difference was between males and females of *T. brevignatha*. There was very little overlap in the chemical profile between the two sexes with 1-methoxy-8,16,22-trimethylhentriacontane (ME 35_A) being the major compound found in female samples while 1-methoxy-6,14,20-trimethylnonacosane (ME 33_B) was the major compound in male samples (SI 6). Nevertheless, in the larger picture, species-specific differences persist strongly despite some sexual differences found in some of the species.

When the relationship between the chemical profiles and underlying phylogeny was visualized using the 3-d traitgram (SI 7), we found that species that were closely related to each other phylogenetically did not exhibit clustering in their chemical profiles and instead appeared to occupy drastically different regions in the NMDS space. This pattern of drastic expansion into distant regions of the NMDS space was particularly noticeable between the co-occurring closely related sister species, *T. trituberculata, T. stelarobusta,* and *T. eurychasma*. A similar but less dramatic pattern was also observed between the co-occurring closely related spiny-leg spiders *T.waikamoi* and *T.kamakou*, as well as between the sister species *T. waikamoi* and *T. brevignatha*.

Co-Occurring Species Have Unique, Non-Overlapping Chemical Profiles

The NMDS results of co-occurring individuals grouped by the two separate sites (Upper Waikamoi & Lower Waikamoi) show again that the chemical profiles are species-specific, but this time show no overlap between the chemical profiles of co-occurring species (Figure 3a, 3b; SI 8). Bray-Curtis distances showed that spiders of the same species had more similar profiles

(Upper: mean = 0.1671371, sd = 0.2725354; Lower: mean = 0.4020551, sd = 0.4354037) than spiders of different species (Upper: mean = 0.9968908, sd = 0.2259125; Lower: mean = 0.9839558, sd =0.07283074), further supporting a species-specific effect on chemical profiles. The differences in mean distance were significant by a t-test (Upper: $df = 1001.4$, $p = < 0.001$; Lower: $df = 827.72$, $p = < 0.001$). PERMANOVA also showed that there were significant differences between the chemical profiles of different species (Upper: ADONIS: $F_{5,95} = 73.068$, $R^2 = 0.85658$, $p = 0.001$; Lower: ADONIS: $F_{3,71} = 37.394$, $R^2 = 0.61241$, $p = 0.001$), with some differences in the level of dispersion of individual chemical profiles between species (Upper: PERMDISP: $F_{5,95} = 12.807$, $p = 0.001$; Lower: PERMDISP: $F_{3,71} = 27.064$, $p = 0.001$). Lastly, pairwise PERMANOVA analyses showed that there were significant differences between the chemical profiles of all co-occurring species pairs. Of these, the Tukey post-hoc test shows that 7 of 15 species pairs in Upper Waikamoi and 2 of 6 species pairs in Lower Waikamoi had similar levels of dispersion (SI 9; SI 10). For these species pairs, differences in chemical profiles by PERMANOVA can be attributed solely to differences in the identity and abundance of the methyl ethers.

Furthermore, when we compare the chemical distance calculated by the number of unshared compounds between co-occurring species pairs and non-overlapping species pairs, we find that co-occurring species had significantly more unshared methyl ethers than species that did not overlap (Allopatric mean = 6.070175, Sympatric mean = 8.285714, *t = -2.3009, df = 31.5, p = 0.02817*) (Figure 4).

Chemical Profiles Differ Between Two Populations

T. stelarobusta, the species that was collected at both the Upper Waikamoi and Lower Waikamoi sites, displayed a population-specific chemical profile despite some overlap between the two sites (Figure 5). PERMANOVA analysis supported a significant difference between the chemical profiles of individuals found at each site (ADONIS: $F_{1,25} = 4.8477$, $R^2 = 0.16242$, $p = 0.027$). Furthermore, there was no significant difference in the intra-population dispersion of the chemical profiles between the two locations (permutest: $F_{1,25} = 4.0327$, $p = 0.051$), thereby indicating that the PERMANOVA result are not affected by dispersion effects.

The relative abundances of the methyl ethers found in the profile of the two populations greatly differed: where chemical profiles from Upper Waikamoi contained significantly higher abundances of 1-methoxy-4,10,16,20,24-pentamethylheptacosane (ME 33_M), 1-methoxy-4,10,16,22,26-pentamethyloctacosane (ME 34_G), and 1-methoxy-4,10,16,22,26-pentamethylnonacosane (ME 35_B) while the Lower Waikamoi profiles contained a relatively higher abundance of 1-methoxy-6,12,18,24,28-pentamethylhentriacontane (ME 37_A) albeit not significant (Figure 5). All other compounds found in the chemical profiles did not have significantly different relative abundances between the two sites. Furthermore, although not significant, the chemical profile of individuals from Upper Waikamoi contained a slightly higher number of methyl ether compounds than profiles of individuals found in Lower Waikamoi (Upper: mean = 7.2 sd = 1.814; Lower: mean = 5.471 sd = 3.319; $F_{1,25} = 2.287$, $p = 0.143$).

Structural Components of Methyl Ethers Differ Between Closely Related Co-Occurring Species

The long chain methyl ethers discussed here differ structurally by chain length as well as number and position of methyl groups along the chain. Their structures were elucidated by combined analysis of their mass spectra, gas chromatographic retention indices and microderivatization of extracts by transformation into cyanides and methyl esters (Stefan Schulz 1997; S. Schulz 2001; Adams et al. 2021). The latter was necessary because the location of methyl groups along the alkyl chain cannot be determined by analysis of the mass spectra of the native methyl ethers. The chemical structure for the 32 most common methyl ethers were thus determined (SI 11; SI 12). Overall, the structural characteristics of the methyl ethers used by each species were relatively consistent except for a few notable differences that existed in certain species (Figure 6). The size of the compounds, defined here as the total number of carbons in the methyl ether including the methoxy group, was significantly different between the co-occurring sister species group, *T. eurychasma, T. stelarobusta,* and *T. trituberculata* where *T. eurychasma* had methyl ethers that were starkly smaller in size (27.480 ± 3.786), *T. stelarobusta* had methyl ethers that were largest (34.950 ± 1.687), and *T. trituberculata* had average sized methyl ethers (32.180 ± 1.623) (Figure 6; SI 13; SI 14). Another notable significant difference in size was found between the sister species pair in the non-web building clade, *T. brevignatha* (34.055 ± 1.660) and *T. waikamoi* (31.275 ± 0.847) (Figure 6; SI 13; SI 14). Both species have only one main methyl ether in their chemical profile and this size difference is most prominent between female chemical profiles where the size of the methyl ether in *T. brevignatha* is 35 carbons whereas in *T. waikamoi* it is 31 carbons in size. In males, the difference becomes 33 carbons vs 31 carbons since male *T. brevignatha* have ME 33_B in their profile as opposed to ME 35_A found in females. Another notable difference was between the closely related co-occurring species *T. waikamoi* (31.275 ± 0.847) and *T. kamakou* (33.983 ± 1.624) with the later having larger methyl ethers in their profile than the former (Figure 6; SI 13; SI 14). As for the number of methyl branches, across all *Tetragnatha* species, the most common number of methyl branches found across all the most common methyl ethers was three branches. However, *T. eurychasma* and *T. stelarobusta* again significantly differed from this trend with *T. eurychasma* having methyl ethers with the fewest number of branches (1.920 ± 0.877) and *T. stelarobusta* having the highest number of branches compared to all other species (4.794 ± 0.514) (Figure 6; SI 13; SI 14). The elongation of the methyl ethers was defined by the number of methylene groups that were present between the first methyl group and the methyl ether oxygen in each compound. The elongation followed a similar pattern as to the number of branches where the starkest difference was seen between *T. eurychasma* and *T. stelarobusta*. With regards to elongation, *T. eurychasma* had the longest elongation (10.80 ± 1.771) and *T. stelarobusta* had the shortest elongation compared to all other species (4.216 ± 1.412) (Figure 6; SI 13; SI 14).

Lastly, the branching pattern, defined here as the ω-methyl branch position of the methyl ethers, showed distinct patterns between certain species and groups. The ancestral state reconstruction of ω-branching positions revealed that even-numbered ω-branch positions are the most common and ancestral form of branching pattern but in the web building clade, odd-numbered ω-branch positions evolved and permeated throughout the clade (SI 15). Across all the species, the most common branch positions were ω-10, ω-16, and ω-22 where methyl ethers with an ω-16 branch were found in all species except for one (Figure 6). *T. stelarobusta* was the only species that

differed entirely from the rest and did not have methyl ethers with any of the branching positions mentioned above. Along with *T. stelarobusta*, "Crater Elongate Forest" was the only other species that lacked methyl ethers with methyl groups at the ω-10 position, whereas *T. brevignatha* and *T. kamakou* join both *T. stelarobusta* and "Crater Elongate Forest", lacking methyl ethers with methyl groups at the ω-22 position (Figure 6).

Hierarchical clustering of the methyl ethers based on maximum common substructure suggested an optimal number of six methyl ether groups (Figure 7; SI 16; SI 17; SI 18). When the methoxy group was accounted for in the analysis, groups were defined mostly by the backbone length rather than methyl branch position (SM 16; SI 17). When the methoxy group was excluded in the analysis, the groups were clustered based on the shared methyl branch positions more strongly than the backbone length (Figure 8; SI 17). As such, groups that had a branched fragment in common tended to be species specific (e.g., Group 3 is specific to *T. stelarobusta*) while the groups with similar backbone length had methyl ethers belonging to many species (Figure 7; SI 18). Interestingly, in both analyses, methyl ethers found in the profile of closely related co-occurring species fell out in vastly different groupings and did not overlap (as seen in the three co-occurring web building sister species *T. eurychasma, T. stelarobusta,* and *T. trituberculata*, as well as the two closely related co-occurring spiny leg species, *T. kamakou* and *T. brevignatha*).

Discussion

Our study shows that (1) males of Hawaiian *Tetragnatha* spiders use chemical cues to discriminate between conspecific and heterospecific females; behavioral analysis using the silk extract of conspecific and heterospecific females showed that males preferred the extract of conspecific females over heterospecific females (Figure 1). And (2) at a given site where multiple species co-occur, these chemical cues are highly species-specific showing no overlap between any of the species; NMDS analysis of the methyl ethers found in the silk extracts differed little between individuals of the same species yet was significantly different between species (Figure 3). Moreover, (3) the chemical structure of the most common methyl ethers found in each species showed significant differences in the fundamental structure of these compounds between closely related co-occurring species (Figure 6).

Male spiders from all six Hawaiian species that were tested in the behavioral assays significantly chose conspecific silk and silk extract over heterospecific silk. Results of the behavioral trials were the same between species from the web building clade and the spiny leg clade, indicating the widespread and fundamental nature of the use of chemicals in species recognition in Hawaiian *Tetragnatha* spiders no matter the change in life history strategies such as the loss of web building behavior. Even when males were tested with heterospecific females from a different clade as their own (as seen in trials with *T. brevignatha* and *T. stelarobusta*), the results were the same as when males were tested with heterospecific females from their own clade (as seen in trials with *T. eurychasma, T. quasimodo, T. trituberculata,* and *T. waikamoi*). This suggests that chemical cues are used to differentiate conspecific mates from all the other *Tetragnatha* species found within a given community no matter the clade or phylogenetic distance.

The components of the chemical profile of each species could be divided into two broad categories: 1) overall composition - the identity, combination, and amount of the methyl ethers found in the profile, and 2) overall structure of the compounds - the size of the methyl ether, the number of methyl branches, the amount of elongation, and the specific ω-methyl branch position of the methyl ethers. Species may differ in either or both categories with no apparent phylogenetic consistency in the nature of these differences.

A key finding of the current study was the fact that overall, there was very little overlap in the methyl ethers used between species and most of the major methyl ethers found in each species was entirely new and unique to that species. Studies conducted on the adaptive radiation of *Heliconius* butterflies, Hawaiian *Drosophila*, and Hawaiian *Laupala* crickets have all shown that the chemical cues between coexisting species are divergent. However, although differences can arise either through changes in the relative amounts of the compounds shared between species or in the existence of new and unique compounds, the former seems to be more prevalent in insects (Alves et al. 2010; Mullen et al. 2007; Stamps and Shaw 2019; Byers et al. 2021; González-Rojas et al. 2020; Darragh et al. 2020). This may not be surprising given that many of these organisms are known to use a multitude of other cues to communicate and may be relying on the combinatory effect of multimodal signaling to recognize appropriate mates, or they have strong dietary and/or host associations that differ despite overlapping geographically (Hoikkala and Welbergen 1995; O'Grady and DeSalle 2018; Mendelson and Shaw 2005; Grace and Shaw 2011; Jiggins et al. 2001; Supple et al. 2014; González-Rojas et al. 2020). Our findings may indicate that *Tetragnatha* spiders heavily depend on chemical cues to communicate as opposed to other means and thereby necessitates the use of finely tuned and highly differentiated chemical profiles.

Chemical profiles between co-occurring Hawaiian *Tetragnatha* spiders were especially distinctive and showed striking differences in the specific identity of methyl ethers found in the silk extracts. In most cases, no methyl ethers were shared between two co-occurring species but if there were overlaps, then it was often only one or two methyl ethers that were shared between the two species. As such, the *Tetragnatha* community found in both Upper and Lower Waikamoi had highly specific combinations of methyl ethers that were never shared between species found from the same site. Furthermore, the specific chemical structure of the methyl ether compounds found in the profile of the spiders differed significantly between closely related co-occurring species. Specifically, the methyl ethers found in the profile of the three co-occurring sister species *T. stelarobusta*, *T. trituberculata*, and *T. eurychasma* differed significantly in the size of the compound, methyl branch number and ω-branch position, and the chain elongation (Figure 6). Hierarchical clustering analysis based on the maximum common substructure of the methyl ethers show that the two co-occurring sister species, *T. stelarobusta* and *T. eurychasma* contain compounds that are structurally significantly different from all other methyl ethers found across all the species and each form a distinct group of structurally distinct *T. stelarobusta* compounds and *T. eurychasma* compounds (Figure 7; SI 18). Less drastic but still significant, differences were also found between the closely related co-occurring species *T. waikamoi* and *T. kamakou* from the non-web building clade. The methyl ether profiles of these two species differed significantly in size and were also grouped in entirely separate maximum common substructure

groupings, indicating the overall structural uniqueness of the compounds between these two species (Figure 7).

While the results from the set of co-occurring taxa showed marked species-specificity, this was not quite the case among species that do not co-occur. Particularly striking is the complete overlap of the chemical profiles between *T. waikamoi*, a non-web building spider from Upper Waikamoi, and *T. filiciphilia*, a web building spider from Lower Waikamoi, that share the same single methyl ether (ME 31_A). Other notable commonalities are between *T. quasimodo* and the two continental *Tetragnatha* spiders *T. versicolor* and *T. pallescens* that all share ME 33_A. Although overall there are only a few methyl ethers shared between any species, it is clear that there is significantly more overlap in chemical profiles between allopatric species than between co-occurring species (Figure 4). On average, 9.35% ± 0.106 of the methyl ethers are shared between the profile of allopatric species pairs as opposed to the 4.10% ± 0.051 between the profile of co-occurring pairs, and the calculated chemical distance is significantly lower for allopatric species pairs than for that of co-occurring pairs (Figure 4).

Lastly, a key component to understanding the mechanisms of reproductive isolation is how recognition cues change and evolve between populations to understand how these cues may eventually lead to reproductive isolation between these populations. In our study we clearly detected differences between populations of a given species. Specifically, for the one species that occurred at the two separate sites we examined (*T. stelarobusta*), there were significant differences between populations in the relative amount of the compounds found in the profiles such that the compounds that differed most from compounds found in the profile of other co-occurring species were most abundant (Figure 5). In the two *T. stelarobusta* populations examined in this study, we see that the chemical profiles are significantly different across the two sites. Specifically, in Upper Waikamoi where *T. stelarobusta* co-occur with the sister species *T. trituberculata* and *T. eurychasma*, we see that the chemical profile of the Upper Waikamoi *T. stelarobusta* has a significantly higher relative abundance of compounds that are structurally divergent to those found in *T. trituberculata*, whereas in *T. stelarobusta* from Lower Waikamoi, they have less of these uniquely structured compounds (Figure 5). Although not significant, specimens of *T. stelarobusta* from Upper Waikamoi had more compounds in their profile than those from Lower Waikamoi.

These results are in agreement with previous work showing that recognition cues between populations vary according to the assemblage of close relatives co-occurring at the given site and the complexity of the signaling environment influenced by these species assemblages (Moritz et al. 2018; Bastian and Jacobs 2015; Lemmon 2009; Wilkins, Seddon, and Safran 2013). It has been shown that at sites where a given species co-occurs with multiple close relatives, the recognition cue of this species diverges significantly from that which it co-occurs with, as compared to populations of the same species at other sites where it co-occurs with distant relatives. Thus, the results support the notion that reinforcing selection may be dictated by the set of closely related species with which a given population co-occurs.

Conclusion

The study highlights the role of chemistry in species recognition among Hawaiian spiders and that the identity of the chemicals is strikingly different among the members of a set of co-occurring taxa within any given community. The methyl ethers found in the chemical profile of co-occurring species differed greatly in the identity, relative amount, and structure of the compound. Moreover, evidence indicates that populations of a given species differ according to the set of taxa contained within the community where a given population is found. This is clearly consistent with the role of these chemicals in fostering recognition. The novelty of our work is that it highlights, for the first time, the mechanism through which chemical diversification may be achieved during adaptive radiation.

Figures & Tables

Fig. 1 – Results for male silk & silk extract choice trials. (A) Silk choice trial and (B) silk extract choice trial setups (not drawn to scale). Males were released at the far end of the center stick. (C) Results of male choice when given a choice between conspecific vs heterospecific female silk in the silk choice trials (Silk - solid bars), and the chemical extract of conspecific vs heterospecific female silk in the silk extract choice trials (Extract - hatched bars). All trials were significant with the degree of significance indicated by the star above each plot (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$). Results for silk choice trials were: *T. brevignatha*: $X^2 = 4.7647$, df = 1, $p = 0.02905$, 13:4; *T. quasimodo*: $X^2 = 7.1176$, df = 1, $p = 0.007633$, 14:3; *T. waikamoi*: $X^2 = 6.25$, df = 1, $p = 0.01242$, 13:3; *T. eurychasma*: $X^2 = 10.714$, df = 1, $p = 0.001063$, 18:3; *T. stelarobusta*: $X^2 = 16.2$, df = 1, $p = <0.001$, 19:1; *T. trituberculata*: $X^2 = 7.1429$, df = 1, $p = 0.007526$, 12:2. Results for silk extract choice trials were: *T. brevignatha*: $X^2 = 9.7826$, df = 1, $p = 0.001762$, 19:4; *T. quasimodo*: $X^2 = 16.133$, df = 1, $p = <0.001$, 26:4; *T. waikamoi*: $X^2 = 14.44$, df = 1, $p = <0.001$, 22:3; *T. eurychasma*: $X^2 = 3.8571$, df = 1, $p = 0.04953$, 15:6; *T. stelarobusta*: $X^2 = 4.1724$, df = 1, $p = 0.04109$, 20:9; *T. trituberculata*: $X^2 = 7.1176$, df = 1, $p = 0.007633$, 14:3.

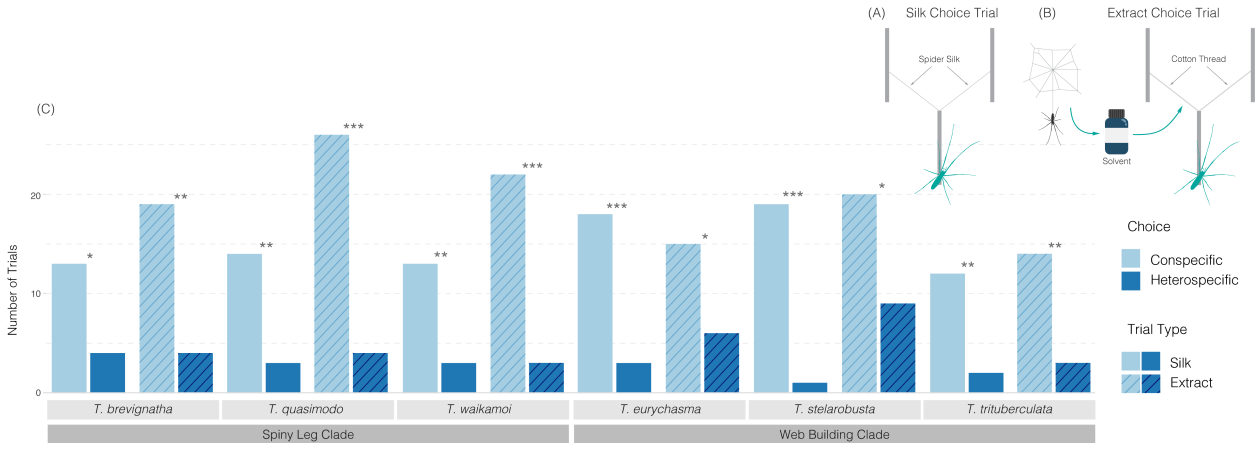


Fig. 2 – Representative chemical profiles of each species. (A) GC-MS chromatograms of representative dragline extracts from females of each species. (B) A closer look at the chromatograms of Hawaiian *Tetragnatha* spiders from the web building clade and (C) Hawaiian *Tetragnatha* spiders from the spiny leg clade with the most abundant methyl ethers labeled with their specific shorthand code. See also SM 2.

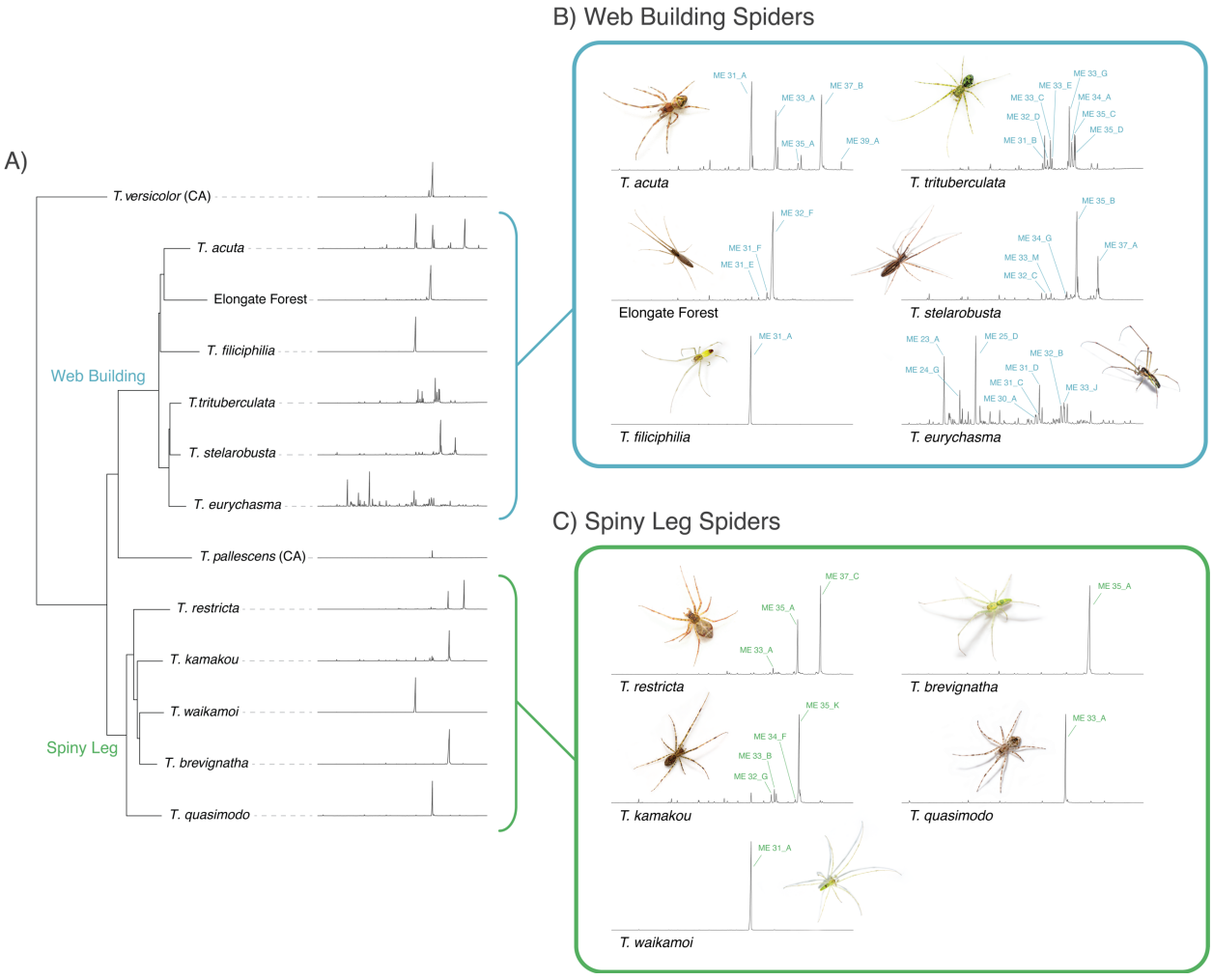


Fig. 3 – Chemical profile of co-occurring individuals and all individuals sampled. NMDS plot of the chemical profiles of the Hawaiian *Tetragnatha* species found in sympatry at (A) Upper Waikamoi (stress = 0.02378555) and at (B) Lower Waikamoi (stress = 0.0307492), as well as a NMDS plot of (C) the chemical profiles of all spider species – 11 Hawaiian *Tetragnatha* species and 2 Californian species (stress = 0.04912099). (D) Location of Upper Waikamoi and Lower Waikamoi on Maui and images of the representative spiders from those two sites and California.

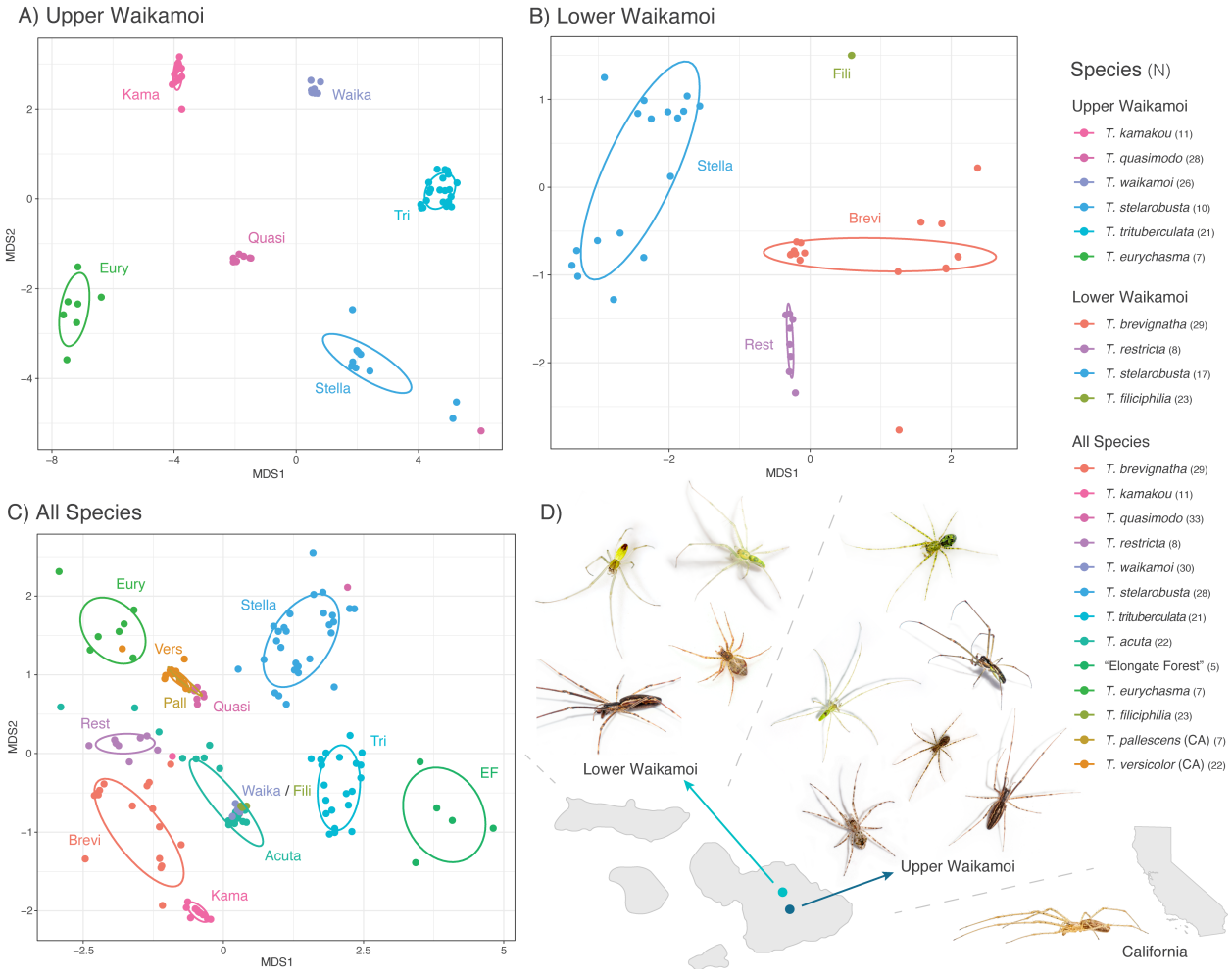


Fig. 4 – Degree of chemical distance between sympatric and allopatric species. Boxplot of the number of differences found in the most common methyl ether profiles of co-occurring and non-co-occurring species pairs. The degree of significance is indicated by the star above the plot (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

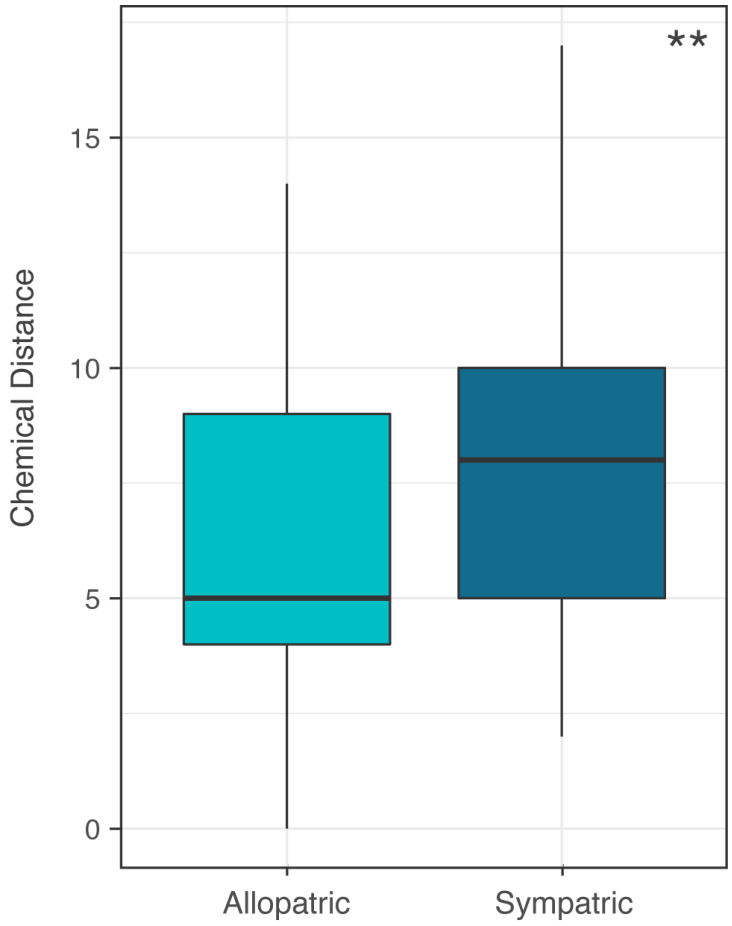


Fig. 5 – Chemical difference between two populations. (A) NMDS plot of the two populations of *T. stelarobusta* collected from Upper Waikamoi and Lower Waikamoi. (B) Box and whisker plot of the relative proportion of specific methyl ethers found in the individual profiles of *T. stelarobusta* from Upper and Lower Waikamoi. The degree of significance is indicated by the star above each plot (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001). The results for each methyl ether compound were: ME 32_C: $F_{1,25} = 2.223$, $p = 0.148$; ME 33_C1/C2: $F_{1,25} = 0.224$, $p = 0.64$; ME 33_M: $F_{1,25} = 6.301$, $p = 0.0189$; ME 34_G: $F_{1,25} = 6.63$, $p = 0.0163$; ME 35_B: $F_{1,25} = 8.188$, $p = 0.0084$; ME 37_A: $F_{1,25} = 4.03$, $p = 0.0556$.

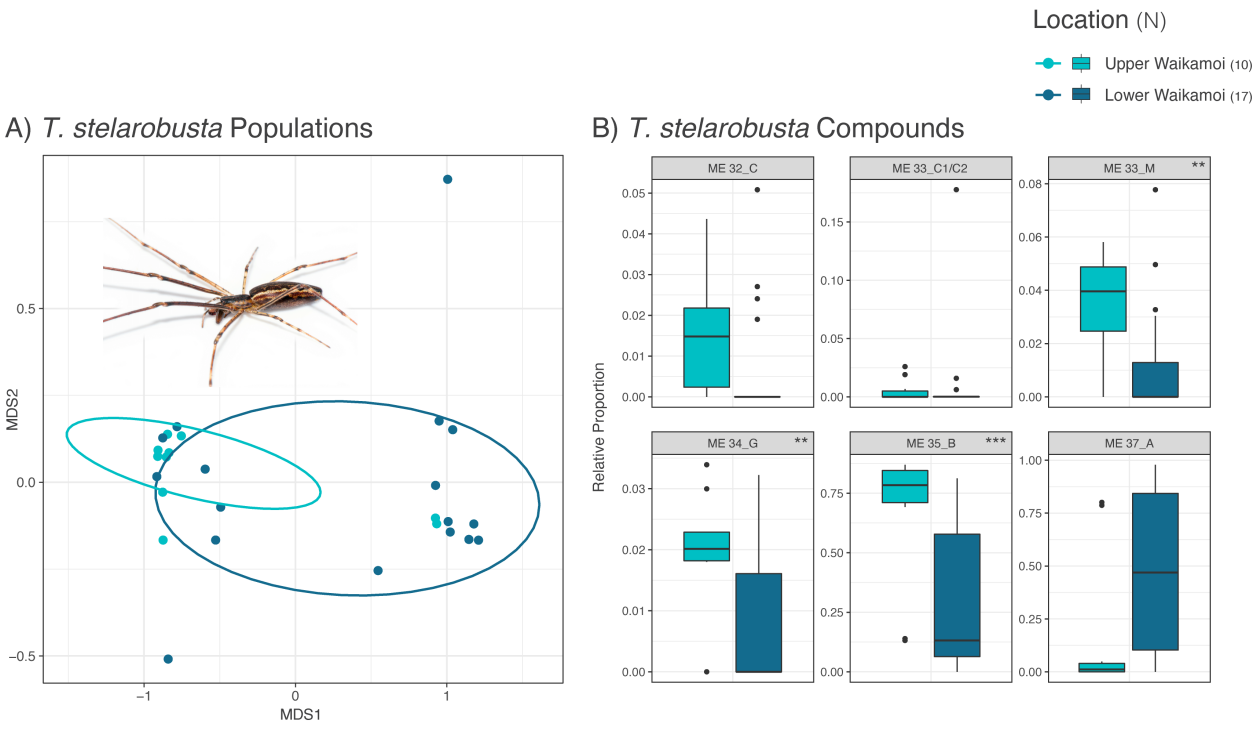


Fig. 6 – Structural characteristics of the most common methyl ethers found in each species. (A) The prominent omega (w) methyl branch patterns of the methyl ethers found in each species is mapped onto a phylogenetic tree where "Type 3" indicates *Tetragnatha* species that have methyl ethers with a branch on position w-10, w-16, and w-22, "Type 2" with a branch on position w-10 and w-16, "Type 1" with a branch on position w-16, and lastly "Type 0" species that do not have any branches on position w-10, w-16, nor w-22. (B) The total number of carbons, including the methoxy group, including the methoxy group, in each compound, (C) the number of methyl branches on each compound, and (D) the elongation amount of each compound is represented in a box and whisker plot where each methyl ether found in an individual profile of a given species is treated as separate points on the plot.

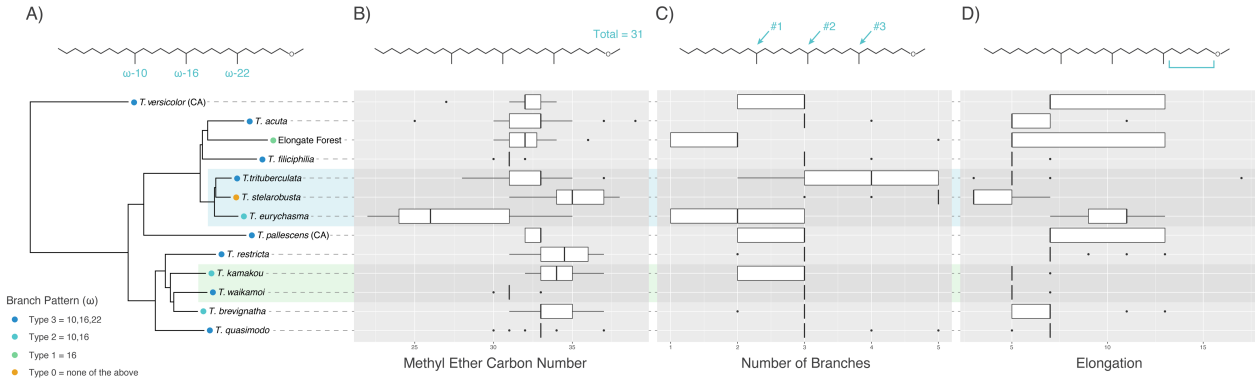
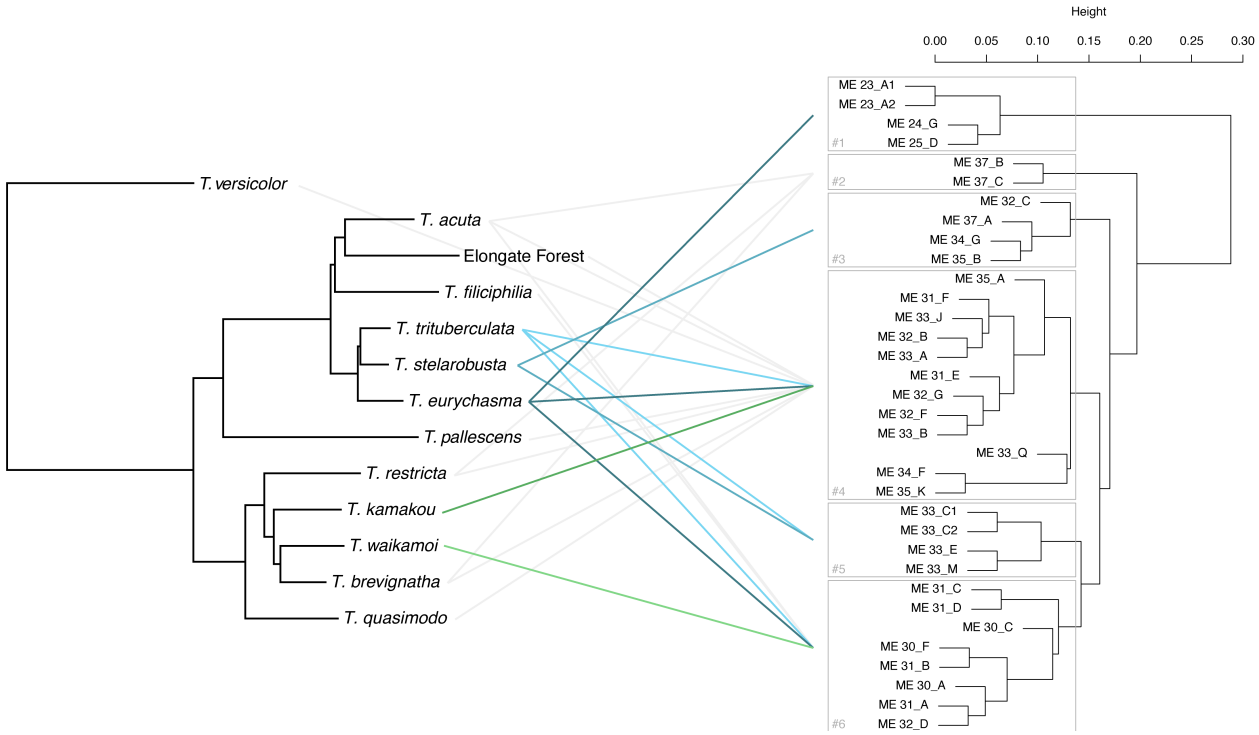


Fig. 7 – Relationship of the methyl ethers based on structural similarity. A visualization of the structural and evolutionary relationship of the most common methyl ether compounds found across the phylogeny. The phylogenetic tree on the left illustrates the evolutionary relationship of the species while the Maximum Common Substructure (MCS) tree on the right illustrates the relationship of structural similarity of all the most common methyl ether compounds found across the species. The gray boxes on the MCS tree indicate the groupings of the methyl ether compounds based on their structural similarity excluding the methoxy group. The lines connecting the phylogenetic tree to the MCS tree indicate the presence of a methyl ether compound from a given MCS grouping in the specific species the line connects to. Blue lines highlight the co-occurring sister species group from the web building clade while the green lines highlight the co-occurring closely related species from the spiny leg clade.



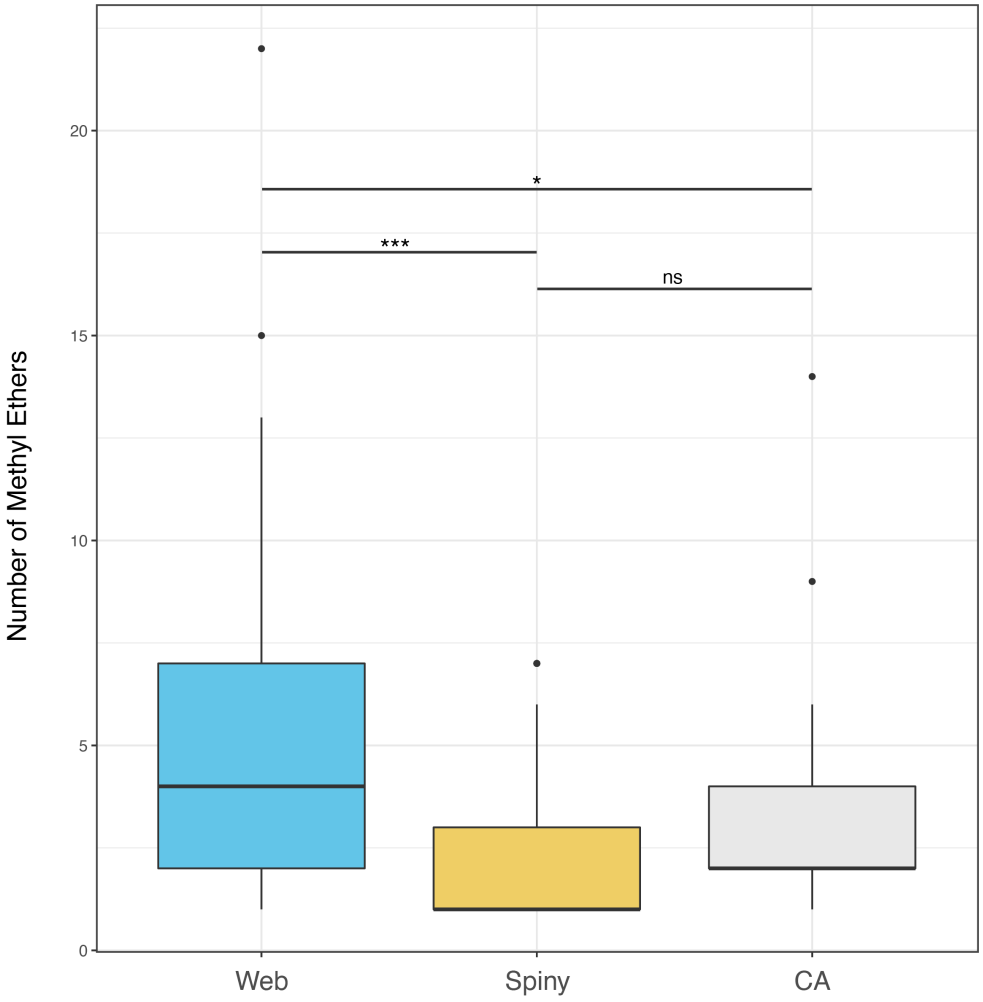
Supplemental Material

SI. 1 – Chemical profile matrix. Matrix containing information about the abundances of the 124 unique methyl ethers found in all the individual extract samples analyzed. Related to figure 2 and figure 3. Data can be found at: https://doi.org/10.6078/D1CB04

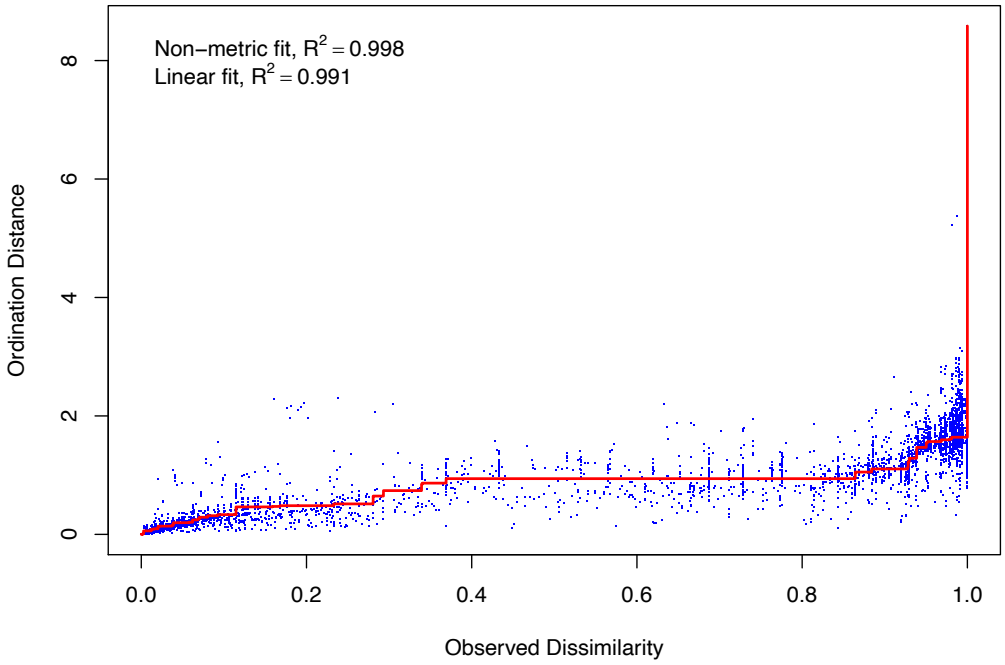
SI. 2 – Most common methyl ethers. A list of all species and their most common methyl ether compounds. Related to figure 2.

Species	Most Common Methyl Ethers
T. acuta	ME 30_C, ME 31_A, ME 33_A, ME 33_B, ME 37_B
T. brevignatha	ME 33_B, ME 35_A, ME 37_B
"Crater Elongate Forest"	ME 31_E, ME 31_F, ME 32_F
T. eurychasma	ME 23_A1, ME 23_A2, ME 24_G, ME 25_D, ME 30_A, ME 31_C, ME 31_D, ME 32_B, ME 33_J
T. filiciphilia	ME 31_A
T. kamakou	ME 32_G, ME 33_B, ME 34_F, ME 35_K
T. pallescens	ME 32_B, ME 33_A
T. quasimodo	ME 33_A
T. restricta	ME 33_A, ME 35_A, ME 37_C
T. stelarobusta	ME 32_C, ME 33_M, ME 34_G, ME 35_B, ME 37_A
T. trituberculata	ME 30_C, ME 30_F, ME 31_B, ME 32_D, ME 33_Q, ME 33_C1, ME 33_C2, ME 33_E
T. versicolor	ME 32_B, ME 33_A
T. waikamoi	ME 31_A

SI. 3 – Number of methyl ethers in the spider profiles. Number of unique methyl ether compounds found in the chemical profile of Hawaiian *Tetragnatha* spiders from the web building clade (Web) and non-web building clade (Spiny), as well as the chemical profile of Californian *Tetragnatha* spiders (CA). Related to figure 2. (ns = not significant, * = $p \leq 0.05$, *** = $p \leq 0.001$)



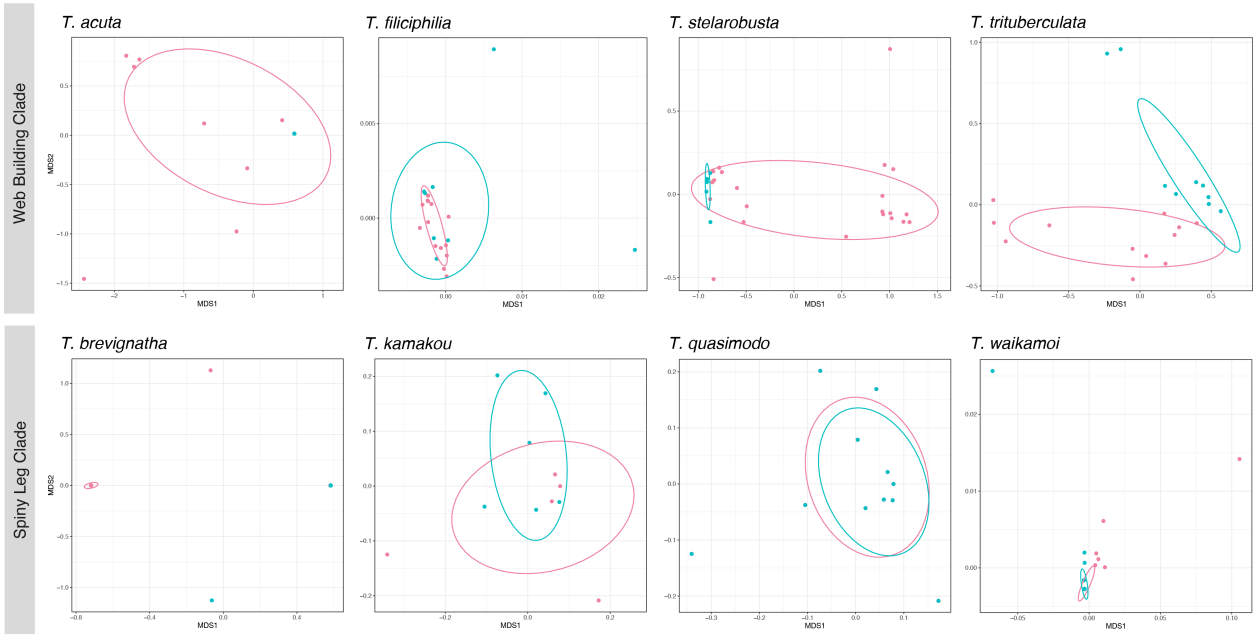
SI. 4 – Goodness of fit plot. Shepard plot to see goodness of fit for the NMDS analysis of all spiders. Related to figure 3.



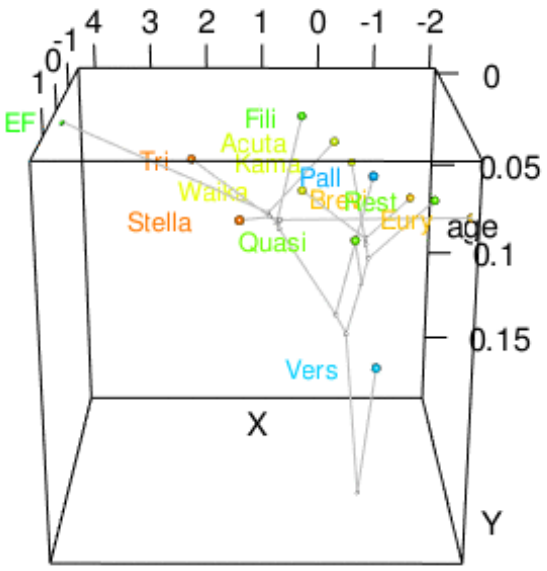
SI. 5 – Average Bray-Curtis distance to median. Results of homogeneity of multivariate dispersions indicating Bray-Curtis distance to median for each species cluster based on chemical profiles of individuals. Related to figure 3.

Species	Average distance to median
T. acuta	0.2689363
T. brevignatha	0.4918154
"Elongate Forest"	0.1551571
T. eurychasma	0.3352235
T. filiciphilia	0.0007601
T. kamakou	0.0961648
T. pallescens	0.0656556
T. quasimodo	0.0655195
T. restricta	0.1201193
T. stelarobusta	0.4390110
T. trituberculata	0.3165952
T. versicolor	0.0918861
T. waikamoi	0.0053748

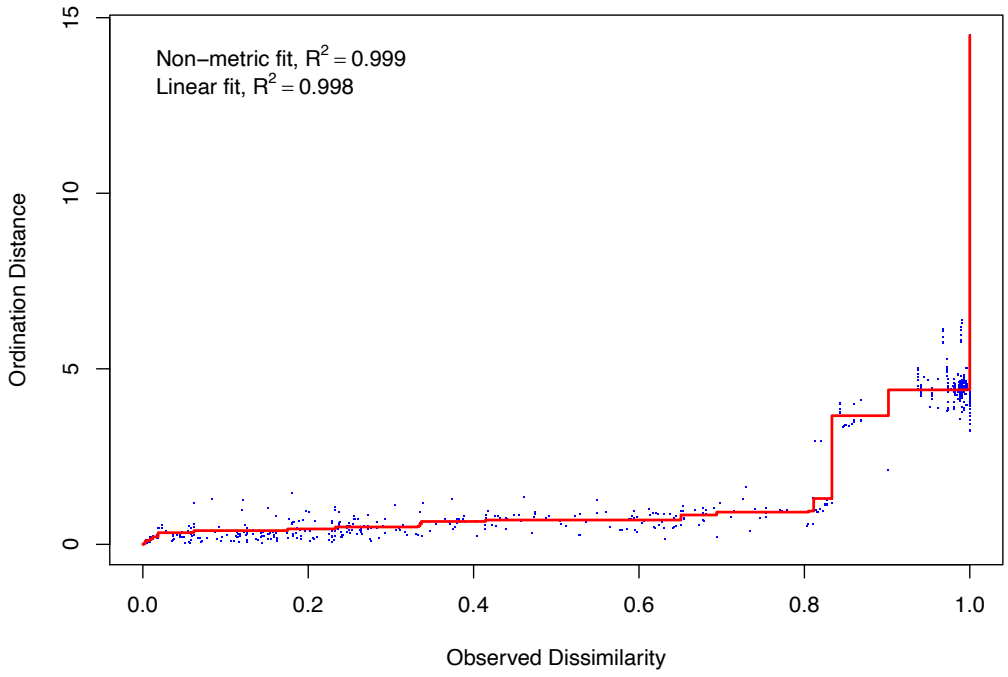
SI. 6 – Sexual differences in chemical profile. NMDS plot of the chemical profiles of male and female Hawaiian *Tetragnatha* spiders in the web building and spiny leg clade. Female = rose colored circles and line. Male = teal-colored circles and line. Results of ADONIS permutation test between sexes for each species were: *T. acuta*: $F_{1,20} = 7.9868$, $R^2 = 0.28538$, $p = 0.002$; *T. filiciphilia*: $F_{1,21} = 3.6622$, $R^2 = 0.14849$, $p = 0.104$; *T. stelarobusta*: $F_{1,26} = 6.0412$, $R^2 = 0.18854$, $p = 0.008$; *T. trituberculata*: $F_{1,19} = 6.0384$, $R^2 = 0.24117$, $p = 0.002$; *T. brevignatha*: $F_{1,27} = 75.557$, $R^2 = 0.73673$, $p = 0.001$; *T. kamakou*: $F_{1,8} = 0.64805$, $R^2 = 0.07494$, $p = 0.726$; *T. quasimodo*: $F_{1,31} = 0.90622$, $R^2 = 0.0284$, $p = 0.162$; *T. waikamoi*: $F_{1,27} = 1.4622$, $R^2 = 0.05137$, $p = 0.176$. Related to figure 3.



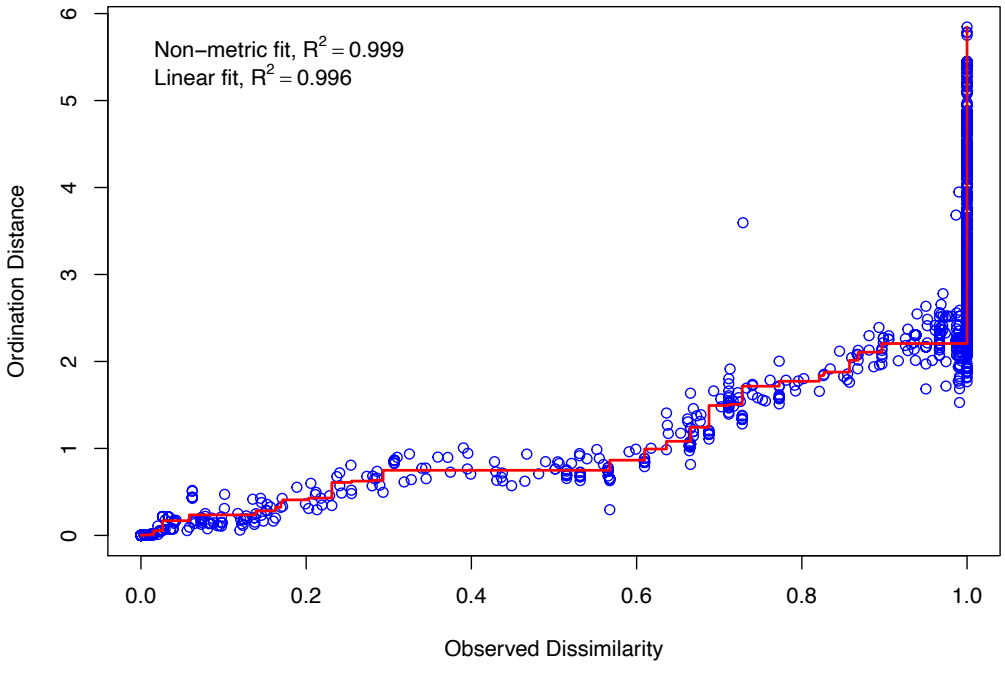
SI. 7 – Three dimensional traitgram. Three dimensional traitgram connecting the phylogenetic relationships between the spiders to their position on the chemical NMDS Plot. The color of each tip of the tree corresponds to the phylogenetic branch length to the nearest node. Related to figure 3.



SI. 8 – Goodness of fit plots for site specific NMDS. Shepard plot to see goodness of fit for the NMDS analysis of (A) Upper Waikamoi spiders and (B) Lower Waikamoi spiders. Related to figure 3.



(A)



(B)

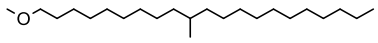
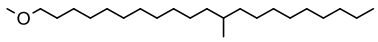
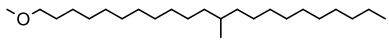
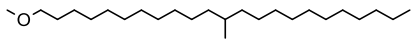
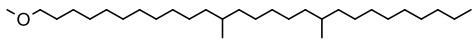
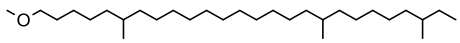
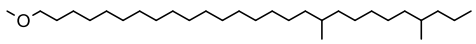
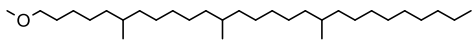
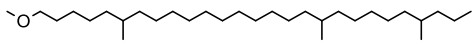
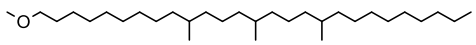
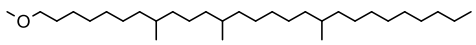
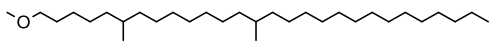
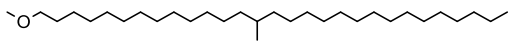
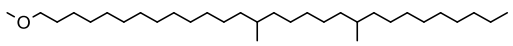
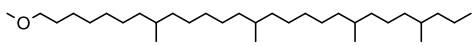
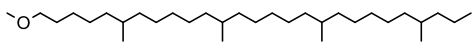
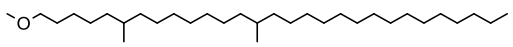
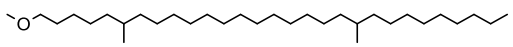
SI. 9 – Pairwise TukeyHSD for Upper Wakamoi. Adjusted p-values of the pairwise TukeyHSD test comparing group dispersions of Upper Waikamoi species. Gray cells indicate statistically significant comparisons. Abbreviations of species names are: "Eury" = *T. eurychasma,* "Kama" = *T. kamakou,* "Quasi" = *T. quasimodo,* "Stella" = *T. stelarobusta,* "Tri" = *T. trituberculata,* "Waika" = *T. waikamoi.* Related to figure 3.

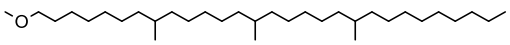
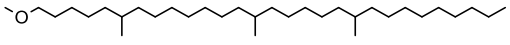
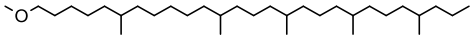
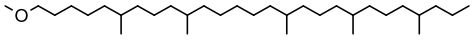
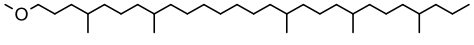
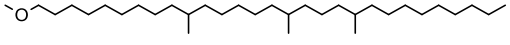
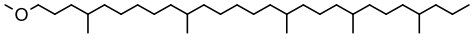
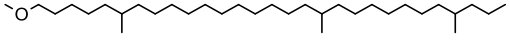
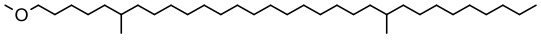
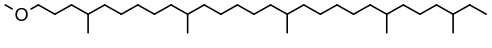
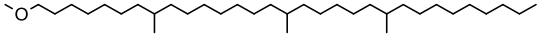
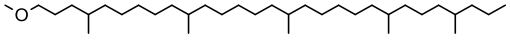
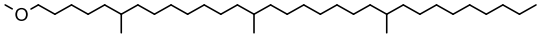
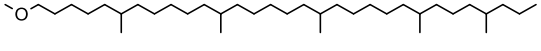
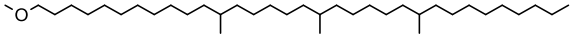
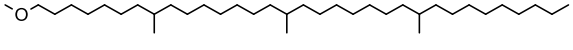
Pairwise Comparison	p-value
Kama-Eury	0.0470165
Quasi-Eury	0.0008434
Stella-Eury	0.8708990
Tri-Eury	0.9997896
Waika-Eury	0.0001568
Quasi-Kama	0.9476002
Stella-Kama	0.3534793
Tri-Kama	0.0105160
Waika-Kama	0.6995178
Stella-Quasi	0.0160440
Tri-Quasi	0.0000017
Waika-Quasi	0.9717383
Tri-Stella	0.8677234
Waika-Stella	0.0030806
Waika-Tri	0.0000001

SI. 10 – Pairwise TukeyHSD for Lower Wakamoi. Adjusted p-values of the pairwise TukeyHSD test comparing group dispersions of Lower Waikamoi species. Gray cells indicate statistically significant comparisons. Abbreviations of species names are: "Brevi" = *T. brevignatha,* "Fili" = *T. filiciphilia,* "Rest" = *T. restricta,* "Stella" = *T. stelarobusta.* Related to figure 3.

Pairwise Comparison	p-value
Fili-Brevi	0.0000000
Rest-Brevi	0.0011480
Stella-Brevi	0.8325941
Rest-Fili	0.6120703
Stella-Fili	0.0000001
Stella-Rest	0.0123136

SI. 11 – General information of the most common methyl ethers identified. The official systematic names and the author appointed code names of the most common methyl ethers for which structure was elucidated for. RI = calculated retention index. Related to figure 6.

Systematic name	Code	RI	Structure
1-methoxy-10-methylhenicosane	ME 23_A1	2367	
1-methoxy-12-methylhenicosane	ME 23_A2	2367	
1-methoxy-12-methyldocosane	ME 24_G	2463	
1-methoxy-12-methyltricosane	ME 25_D	2566	
1-methoxy-12,18-dimethylheptacosane	ME 30_A	2988	
1-methoxy-6,18,24-trimethylheptacosane	ME 30_C	2929	
1-methoxy-18,24-dimethylheptacosane	ME 30_F	3036	
1-methoxy-6,12,18-trimethylheptacosane	ME 31_A	3055	
1-methoxy-6,18,24-trimethylheptacosane	ME 31_B	3081	
1-methoxy-10,14,18-trimethylheptacosane	ME 31_C	3012	
1-methoxy-8,12,18-trimethylheptacosane	ME 31_D	3019	
1-methoxy-6,14-dimethyloctacosane	ME 31_E	3093	
1-methoxy-14-methylnonacosane	ME 31_F	3158	
1-methoxy-14,20-dimethylnonacosane	ME 32_B	3217	
1-methoxy-8,14,20,24-tetramethylheptacosane	ME 32_C	3080	
1-methoxy-6,12,18,24-tetramethylheptacosane	ME 32_D	3096	
1-methoxy-6,14-dimethylnonacosane	ME 32_F	3205	
1-methoxy-6,20-dimethylnonacosane	ME 32_G	3194	

Systematic name	Code	RI	Structure
1-methoxy-8,14,20-trimethylnonacosane	ME 33_A	3255	
1-methoxy-6,14,20-trimethylnonacosane	ME 33_B	3226	
1-methoxy-6,12,16,20,24-pentamethylheptacosane	ME 33_C1	3127	
1-methoxy-6,10,16,20,24-pentamethylheptacosane	ME 33_C2	3127	
1-methoxy-4,8,16,20,24-pentamethylheptacosane	ME 33_E	3139	
1-methoxy-10,16,20-trimethylnonacosane	ME 33_J	3213	
1-methoxy-4,10,16,20,24-pentamethylheptacosane	ME 33_M	3106	
1-methoxy-6,18,26-trimethylnonacosane	ME 33_Q	3271	
1-methoxy-6,22-dimethylhentriacontane	ME 34_F	3419	
1-methoxy-4,10,16,22,26-pentamethyloctacosane	ME 34_G	3239	
1-methoxy-8,16,22-trimethylhentriacontane	ME 35_A	3456	
1-methoxy-4,10,16,22,26-pentamethylnonacosane	ME 35_B	3344	
1-methoxy-6,14,22-trimethylhentriacontane	ME 35_K	3455	
1-methoxy-6,12,18,24,28-pentamethylhentriacontane	ME 37_A	3546	
1-methoxy-12,18,24-trimethyltritriacontane	ME 37_B	3610	
1-methoxy-8,16,24-trimethyltritriacontane	ME 37_C	3625	

SI. 12 – Derivatization information of the most common methyl ethers. The chemical structure of the most common methyl ethers found across all species with relevant descriptors of how the structure was implied from the derivatives created from the iodination using cyanide (IC) and oxidation (Oxi) reactions. Related to figure 6. Data can be found at: https://doi.org/10.6078/D1CB04

SI. 13 – Average values of key structural components in the methyl ethers. The means and standard deviations of the total carbon number of all the methyl ethers found in the profile of each species, the number of methyl branches on the most common methyl ethers found in each species, and the elongation amount of the most common methyl ethers found in each species. Each methyl ether found in an individual profile were treated separately and the mean/sd were calculated based on the compounds found across all individuals of a given species. Related to figure 6.

Species	Methyl Ether Carbon Number	Number of Methyl Branches	Elongation
T. acuta	32.833 ± 2.788	3.052 ± 0.223	6.207 ± 1.871
T. brevignatha	34.055 ± 1.660	2.959 ± 0.200	7.082 ± 2.414
"Elongate Forest"	31.955 ± 1.430	2.067 ± 1.280	7.667 ± 3.904
T. eurychasma	27.480 ± 3.786	1.920 ± 0.877	10.080 ± 1.771
T. filiciphilia	30.962 ± 0.344	3.038 ± 0.196	5.154 ± 0.543
T. kamakou	33.983 ± 1.624	2.524 ± 0.505	5.048 ± 0.309
T. pallescens	32.636 ± 0.505	2.636 ± 0.505	9.182 ± 3.027
T. quasimodo	32.851 ± 1.179	3.125 ± 0.404	6.750 ± 0.670
T. restricta	34.500 ± 1.767	2.857 ± 0.356	7.786 ± 1.833
T. stelarobusta	34.950 ± 1.687	4.794 ± 0.514	4.216 ± 1.412
T. trituberculata	32.180 ± 1.623	3.965 ± 1.021	5.684 ± 3.257
T. versicolor	32.143 ± 1.458	2.524 ± 0.505	9.857 ± 3.033
T. waikamoi	31.275 ± 0.847	3.000 ± 0.000	5.154 ± 0.540

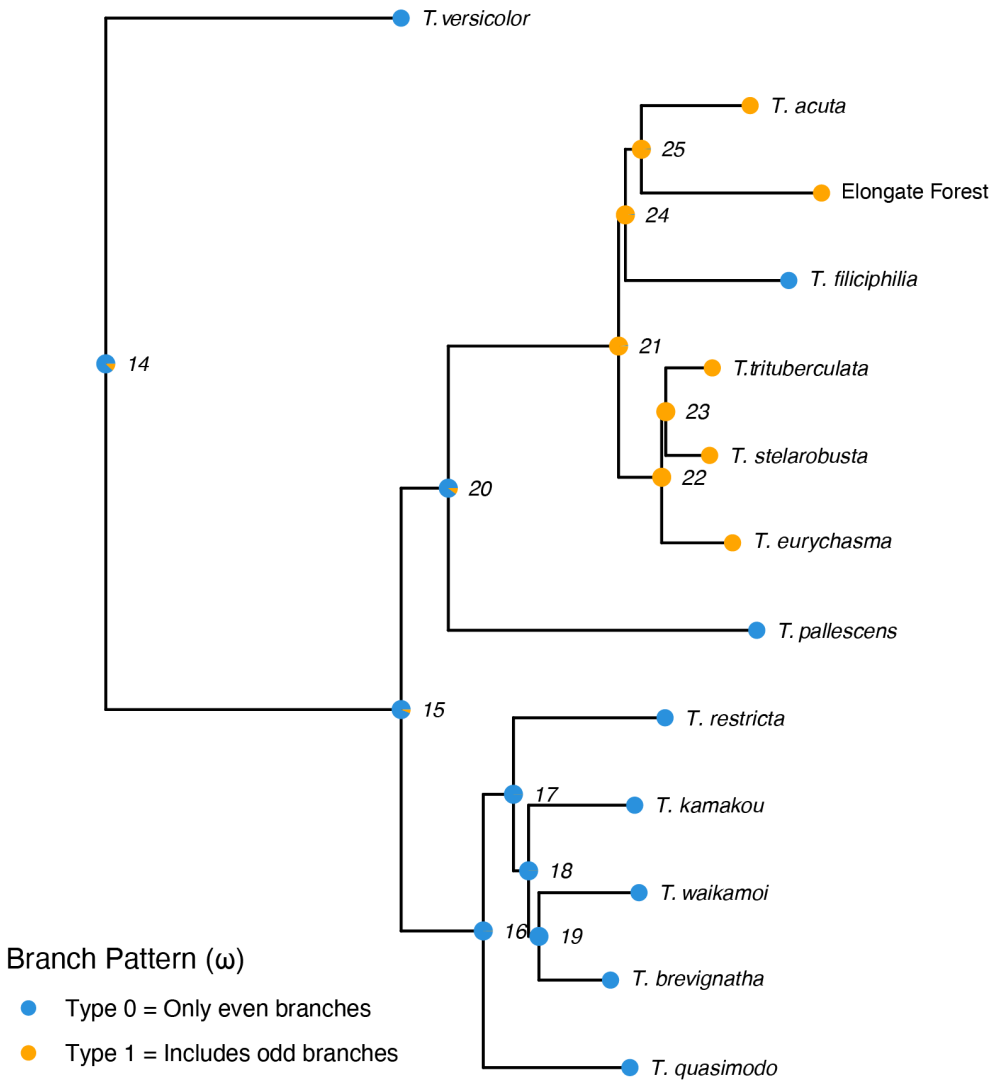
SI. 14 – Pairwise comparisons of the key structural components in methyl ethers. Adjusted p-values of the pairwise TukeyHSD test for each category. Overall ANOVA results were: Methyl Ether Carbon Number: $F_{12,832} = 76.05$, $p = <0.001$; Number of Methyl Branches: $F_{12,603} = 97.31$, $p = <0.001$; Elongation: $F_{12,603} = 36.99$, $p = <0.001$. Gray cells indicate statistically significant comparison. Abbreviations of species names are: "Acuta" = *T. acuta,* "Brevi" = *T. brevignatha,* "EF" = "Elongate Forest", "Eury" = *T. eurychasma,* "Fili" = *T. filiciphilia,* "Kama" = *T. kamakou,* "Pall" = *T. pallescens,* "Quasi" = *T. quasimodo,* "Rest" = *T. restricta,* "Stella" = *T. stelarobusta,* "Tri" = *T. trituberculata,* "Vers" = *T. versicolor,* "Waika" = *T. waikamoi*. Related to figure 6.

Pairwise Comparison	Methyl Ether Carbon Number	Number of Methyl Branches	Elongation
Brevi-Quasi	0.098	0.992	1.000
Waika-Quasi	0.012	1.000	0.060
Kama-Quasi	0.141	0.002	0.025
Rest-Quasi	0.008	0.891	0.774
Pall-Quasi	1.000	0.551	0.056
Eury-Quasi	< 0.0001	< 0.0001	< 0.0001
Stella-Quasi	< 0.0001	< 0.0001	< 0.0001
Tri-Quasi	0.673	< 0.0001	0.272
Fili-Quasi	0.006	1.000	0.155
EF-Quasi	0.864	< 0.0001	0.975
Acuta-Quasi	1.000	1.000	0.992
Vers-Quasi	0.864	0.002	< 0.0001
Waika-Brevi	< 0.0001	1.000	0.003
Kama-Brevi	1.000	0.062	0.001
Rest-Brevi	0.997	1.000	0.979
Pall-Brevi	0.599	0.951	0.163
Eury-Brevi	< 0.0001	< 0.0001	< 0.0001
Stella-Brevi	0.138	< 0.0001	< 0.0001
Tri-Brevi	< 0.0001	< 0.0001	0.011
Fili-Brevi	< 0.0001	1.000	0.017
EF-Brevi	0.002	< 0.0001	0.999
Acuta-Brevi	0.038	1.000	0.681
Vers-Brevi	< 0.0001	0.062	< 0.0001
Kama-Waika	< 0.0001	0.044	1.000
Rest-Waika	< 0.0001	1.000	< 0.0001
Pall-Waika	0.706	0.904	< 0.0001
Eury-Waika	< 0.0001	< 0.0001	< 0.0001
Stella-Waika	< 0.0001	< 0.0001	0.522
Tri-Waika	0.290	< 0.0001	0.984
Fili-Waika	1.000	1.000	1.000
EF-Waika	0.985	< 0.0001	0.010

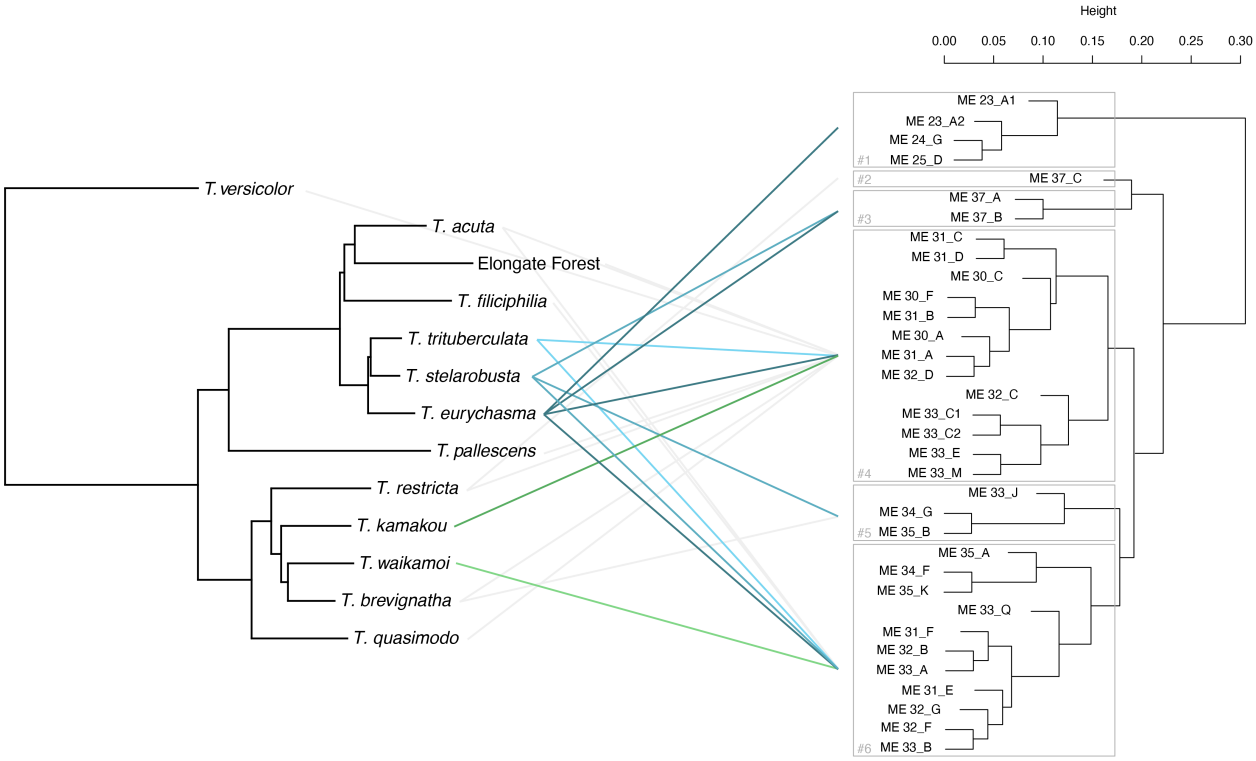
Pairwise Comparison	Methy Ether Carbon Number	Number of Methyl Branches	Elongation
Acuta-Waika	0.005	1.000	0.488
Vers-Waika	0.680	0.044	< 0.0001
Rest-Kama	0.989	0.630	< 0.0001
Pall-Kama	0.670	1.000	< 0.0001
Eury-Kama	< 0.0001	0.001	< 0.0001
Stella-Kama	0.056	< 0.0001	0.672
Tri-Kama	< 0.0001	< 0.0001	0.922
Fili-Kama	< 0.0001	0.065	1.000
EF-Kama	0.003	0.454	0.005
Acuta-Kama	0.058	0.003	0.292
Vers-Kama	< 0.0001	1.000	< 0.0001
Pall-Rest	0.215	0.999	0.846
Eury-Rest	< 0.0001	< 0.0001	0.001
Stella-Rest	0.987	< 0.0001	< 0.0001
Tri-Rest	< 0.0001	< 0.0001	< 0.0001
Fili-Rest	< 0.0001	0.998	0.001
EF-Rest	< 0.0001	0.007	1.000
Acuta-Rest	0.002	0.983	0.082
Vers-Rest	< 0.0001	0.630	0.007
Eury-Pall	< 0.0001	0.042	0.991
Stella-Pall	0.010	< 0.0001	< 0.0001
Tri-Pall	1.000	< 0.0001	< 0.0001
Fili-Pall	0.463	0.870	< 0.0001
EF-Pall	0.999	0.551	0.870
Acuta-Pall	1.000	0.743	0.002
Vers-Pall	1.000	1.000	0.999
Stella-Eury	< 0.0001	< 0.0001	< 0.0001
Tri-Eury	< 0.0001	< 0.0001	< 0.0001
Fili-Eury	< 0.0001	< 0.0001	< 0.0001
EF-Eury	< 0.0001	1.000	0.011
Acuta-Eury	< 0.0001	< 0.0001	< 0.0001
Vers-Eury	< 0.0001	0.001	1.000
Tri-Stella	< 0.0001	< 0.0001	< 0.0001
Fili-Stella	< 0.0001	< 0.0001	0.755
EF-Stella	< 0.0001	< 0.0001	< 0.0001
Acuta-Stella	< 0.0001	< 0.0001	< 0.0001
Vers-Stella	< 0.0001	< 0.0001	< 0.0001
Fili-Tri	0.139	< 0.0001	0.996
EF-Tri	1.000	< 0.0001	0.051
Acuta-Tri	0.508	< 0.0001	0.958
Vers-Tri	1.000	< 0.0001	< 0.0001

Pairwise Comparison	Methy Ether Carbon Number	Number of Methyl Branches	Elongation
EF-Fili	0.874	< 0.0001	0.023
Acuta-Fili	0.003	1.000	0.696
Vers-Fili	0.387	0.065	< 0.0001
Acuta-EF	0.839	< 0.0001	0.503
Vers-EF	1.000	0.454	0.046
Vers-Acuta	0.814	0.003	< 0.0001

SI. 15 – Ancestral state reconstruction of methyl branch patterns. An ancestral state reconstruction of the w-methyl branch pattern found in each species. "Type 0" indicates *Tetragnatha* species that only had methyl ethers with even numbered w-methyl branches, whereas "Type 1" indicates species that had some methyl ethers with odd numbered w-methyl branches. Posterior probabilities for "Type 0" and "Type 1" respectively for each node were: Node 14: 0.879, 0.121; Node 15: 0.940, 0.060; Node 16: 0.995, 0.005; Node 17: 0.999, 0.001; Node 18: 1.000, 0.000; Node 19:1.000, 0.000; Node 20: 0.897, 0.103; Node 21: 0.018, 0.982; Node 22: 0.000, 1.000; Node 23: 0.000, 1.000; Node 24: 0.017, 0.983; Node 25: 0.015, 0.985. Related to figure 6.



SI. 16 – Relationship of methyl ethers based on structural similarity of the entire methyl ether. A visualization of the structural and evolutionary relationship of the most common methyl ether compounds found across the phylogeny. The phylogenetic tree on the left illustrates the evolutionary relationship of the species while the Maximum Common Substructure (MCS) tree on the right illustrates the relationship of structural similarity of all the most common methyl ether compounds found across the species. The gray boxes on the MCS tree indicate the groupings of the methyl ether compounds based on their structural similarity including the methoxy group. The lines connecting the phylogenetic tree to the MCS tree indicate the presence of a methyl ether compound from a given MCS grouping in the specific species the line connects to. Blue lines highlight the co-occurring sister species group from the web building clade while the green lines highlight the co-occurring closely related species from the spiny leg clade. Related to figure 7.



SI. 17 – Maximum common substructure clustering of methyl ethers. Description of groups found by hierarchical clustering of the most common methyl ethers based on maximum common substructure including the methoxy group. Related to figure 7.

Group #	Methyl Ethers	Clusterwise Jaccard bootstrap mean	Common structural features	Species w/ Methyl Ether in this Group
1	23_A1, 23_A2, 24_G, 25_D	0.9833333	Short backbone length (21-23), Branch: 12 or ω-12	*T. eurychasma*
2	37_C	0.5543333	-	*T. restricta*
3	33_J, 34_G, 35_B	0.7041041	Backbone: 28-29 Branch: ω-14, 20	*T. stelarobusta, T. eurychasma*
4	35_A, 31_F, 32_B, 33_A, 31_E, 32_G, 32_F, 33_B, 33_Q, 34_F, 35_K	0.7750475	Backbone: 28-31	*T. acuta, T. brevignatha, T. eurychasma,* "EF"*, T. kamakou, T. pallescens, T. quasimodo, T. restricta, T. trituberculata, T. versicolor*
5	37_A, 37_B	0.6301469	Backbone: 31-33	*T. stelarobusta, T. brevignatha*
6	31_C, 31_D, 30_C, 30_F, 31_B, 30_A, 31_A, 32_D, 32_C, 33_C1, 33_C2, 33_M, 33_E	0.7082247	Backbone: 25-27 Branch: 18	*T. acuta, T. eurychasma, T. filiciphilia, T. trituberculata, T. waikamoi, T. stelarobusta*

SI. 18 – Maximum common substructure clustering of methyl ethers without the methoxy group Description of groups found by hierarchical clustering of the most common methyl ethers based on maximum common substructure excluding the methoxy group. Related to figure 7.

Group #	Methyl Ethers	Clusterwise Jaccard bootstrap mean	Common structural features	Species w/ Methyl Ether in this Group
1	23_A1, 23_A2, 24_G, 25_D	0.9840000	Short backbone length (21-23) Branch: 12 or ω-12	*T. eurychasma*
2	37_B, 37_C	0.6798040	Backbone: 33 Branch: ω-18	*T. acuta, T. brevignatha, T. restricta*
3	32_C, 34_G, 35_B, 37_A	0.6602234	Backbone: 27-31 Branch: ω-4,8,14,20	*T. stelarobusta*
4	35_A, 31_F, 33_J, 32_B, 33_A, 31_E, 32_G, 32_F, 33_B, 33_Q, 34_F, 35_K	0.8176298	Backbone: 28-31	*T. acuta, T. brevignatha, T. eurychasma,* "EF"*, T. kamakou, T. pallescens, T. quasimodo, T. restricta, T. trituberculata, T. versicolor*
5	33_C1, 33_C2, 33_M, 33_E	0.6911976	Backbone: 27 Branch: ω-4,8,12	*T. trituberculata, T. stelarobusta*
6	31_C, 31_D, 30_C, 30_F, 31_B, 30_A, 31_A, 32_D	0.7073252	Backbone: 25-27 Branch: 18	*T. acuta, T. eurychasma, T. filiciphilia, T. trituberculata, T. waikamoi*

SI. 19 – Silk and silk extract choice test combinations. The combination of heterospecific species used in the silk and silk extract choice trials for each species tested. All heterospecific species are from the same site and the same clade as the subject species except for those of *T. brevignatha* and *T. stelarobusta* which are from the same site (Lower Waikamoi) but are spiny leg and web building spiders respectively. Related to figure 1.

Subject Species	**Heterospecific Species Tested**
T. brevignatha	*T. stelarobusta* (Lower Waikamoi)
T. eurychasma	*T. trituberculata*
T. quasimodo	*T. waikamoi*
T. stelarobusta (Lower Waikamoi)	*T. brevignatha*
T. trituberculata	*T. eurychasma*
T. waikamoi	*T. quasimodo*

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In the next chapter, I investigate the desiccation resistance and cuticular chemicals of the Hawaiian *Tetragnatha* spiders that inhabit dry and wet habitats to explore the role of methyl ether compounds in desiccation resistance and mate recognition.

Chapter 3

Desiccation Resistance and the Role of Cuticular Lipids in the Adaptive Radiation of Hawaiian *Tetragnatha* Spiders (Araneae: Tetragnathidae)

Seira A. Adams · Tyler Moy · Kevin Roberts · Rosemary G. Gillespie

Abstract

Factors involved in facilitating the process of divergence and speciation have garnered much attention in evolutionary biology and certain traits often referred to as 'magic traits' have been shown to accelerate this process tremendously by playing a role in both divergent selection and assortative mating. Cuticular hydrocarbons in insects are well known to act as magic traits by functioning in both mate recognition and desiccation resistance. Nevertheless, not all arthropods use cuticular hydrocarbons to identify mates and the potential for non-cuticular hydrocarbon compounds to act as magic traits have yet to be explored. Using desiccation assays and gas chromatography-mass spectrometry, we explore the desiccation resistance and cuticular lipids of closely related Hawaiian *Tetragnatha* spiders that inhabit drastically different wet and dry habitats as well as have differing life history strategies (web building and cursorial). Unsurprisingly, species that inhabit drier habitats survived the longest in the desiccation assay, but no desiccation resistance differences were found between the two life history strategies. The cuticular lipid composition of the spiders consisted mostly of methyl ethers, compounds known to be used in mate recognition but, interestingly, these methyl ethers did not correspond to the desiccation differences found between the spider species. Instead, hydrocarbons that were found only in relatively small portions across the cuticle, strongly correlated with desiccation resistance. These findings provide key insight into the role of cuticular lipids in accelerated reproductive isolation in the context of divergent ecological selection.

Introduction

A major focus in evolutionary biology is the identification of factors that facilitate and accelerate speciation, with a specific emphasis on the interplay between gene flow and selection (Price 2008; Butlin and Ritchie 2009; Butlin, Galindo and Grahame 2008; Nosil 2012) and the genomics of traits involved in divergence, with and without gene flow (Feder, Egan and Nosil 2012; Feder et al. 2013). The primary issue is that, when isolation is not complete, gene flow and recombination randomize associations between genes under divergent selection and those that lead to non-random mating. However, when these two forces of divergent selection and non-random mating are influenced by the same trait, the process of reproductive isolation is greatly accelerated (Nosil 2012; Servedio and Kopp 2012; Seehausen and Magalhaes 2011). These traits – often referred to as 'magic traits' – can be a powerful factor in generating isolation, yet it is unclear whether this is a common phenomenon in rapidly diverging lineages or whether the two roles of divergent selection and non-random mating are controlled by separate traits.

Lipids found on the cuticle of insects, specifically cuticular hydrocarbons (CHCs), have been found to play a role in both divergent selection and non-random mating. CHCs are long-chain aliphatic hydrocarbons found on the cuticular surface of many arthropods and have been shown to play a role in adjusting the permeability of water through the insect's cuticle (Gibbs and Rajpurohit 2010). Studies conducted on *Drosophila* have shown that the cuticular hydrocarbon profile of a population changes in response to a hotter environment, increasing the relative amounts of compounds that are better suited for waterproofing. This then causes partial reproductive isolation because the cuticular hydrocarbon profile is used for mate recognition (Chung et al. 2014; Chung and Carroll 2015). Specifically, Chung et al (2014) found that silencing genes encoding the methyl-branched cuticular hydrocarbon, a *microsomal fatty acid synthase* gene, in *Drosophila serrata* decreased production of methyl-branched alkanes (but not straight chained alkanes or alkenes) which then led to a decrease in desiccation resistance and male mating success. Similar findings have been shown in leaf beetles (Otte, Hilker and Geiselhardt 2015) and wasps (Buellesbach et al. 2013), where cuticular hydrocarbons seem to play a role in both mediating desiccation tolerance as well as communication between individuals and colonies (Menzel, Zumbusch and Feldmeyer 2018; Buellesbach et al. 2018). Thus, CHCs can be considered a classic example of a magic trait, as selection will act on them simultaneously to: (a) prevent desiccation and (b) allow mate recognition (Butterworth et al. 2020).

Nevertheless, while all arthropods have an epicuticular lipid layer, not all have a diversity of cuticular hydrocarbons and not all use CHCs to identify mates. Among spiders in particular, both the cuticle and the silk are covered by a lipid layer, which protects the spider from desiccation as well as serving for communication (Trabalon and Bagnères 2010). This layer comprises mostly long-chain aliphatic hydrocarbons and fatty acids, with smaller amounts of methyl esters, long-chain aliphatic alcohols and aldehydes, glycerides and cholesterol. However, the hydrocarbons found in spiders are often not species specific and not found to be involved in mate recognition (with a few exceptions), possibly because a pattern of hydrocarbons may be susceptible to contamination by cuticular hydrocarbons from insect prey (Schulz 2004). Instead, methyl-branched long-chain alkyl methyl ethers which are also abundant on the cuticle, show a diverse species-specific pattern in spiders and appear to be used for mate recognition (Schulz 2004;

Gerbaulet et al 2022; Trabalon and Garcia 2021). The current study focuses specifically on the epicuticular wax of spiders to understand whether the compounds that comprise the lipid layer, though differing fundamentally from insects, can act in both mate recognition and environmental adaptation in the same way as insects.

The adaptive radiation of spiders in the genus *Tetragnatha* is one of the most well-known adaptive radiations in the Hawaiian Islands and comprises over 50 different species found in a variety of habitats, though almost exclusively within wet or mesic forest environments (Kennedy et al. 2022), across the archipelago. Recent evidence suggests that, like other spiders, Hawaiian *Tetragnatha* use chemical cues to identify suitable mates, and these cues are primarily made up of a mixture of various methyl ether compounds (Adams et al. 2021). The major question is, to what extent do these same chemicals control water loss according to the lifestyle and habitat of a given taxon? The Hawaiian *Tetragnatha* provide an ideal system for examining this question because there are marked differences in both lifestyle and habitat among different members of the radiation. First, in terms of lifestyle, the lineage is divided into two clades: the "web-building" clade that builds orb-shaped webs in the vegetation and the "spiny leg" clade that has abandoned web building and lives a cursorial lifestyle hunting prey without webs (Kennedy et al. 2022; Gillespie 1991; Gillespie 1992). The difference in lifestyle inevitably exposes taxa to different water needs. For example, differences in overall mobility can change the amount of water lost through respiration with more active cursorial spiders lose more water than sedentary spiders (Chown 2002), while web-building spiders can take in more water when ingesting and recycling their webs that have become saturated with moisture from the environment (Opell 2020). Second, in terms of habitat, while most species are limited to wet forest, one lineage in the web-building clade is found exclusively in dry forest and at high elevation sites on the islands where the conditions can be almost desert-like with very little rainfall compared to the wet forests. The existence of closely related species that have different lifestyle traits (cursorial versus web-building), as well as some that have adapted to dry environments, provides an ideal system with which to examine how desiccation stress may affect the same chemicals as those involved in mate recognition.

To study the role of cuticular chemicals in alleviating desiccation stress while also serving as mate recognition cues, we set out to test the following hypotheses: Assuming that species living in more arid environments, as well as those with a more cursorial lifestyle, are more resistant to desiccation compared to the closely related wet forest inhabiting counterparts, we hypothesized that, like the cuticular hydrocarbons in *Drosophila* and ants, the methyl ethers used in mate recognition in spiders are also involved in mediating desiccation resistance. Alternatively, the two functions of desiccation resistance and mate recognition may be performed by separate traits. In this scenario, we hypothesize that the methyl ethers found on the spider cuticle are specialized for mate recognition while a different class of chemicals is involved in desiccation resistance. We examined these two alternative hypotheses by measuring standard physiological responses to desiccation resistance (tolerance to desiccation and abundance of metabolic lipid stores) and then analyzed the composition of the cuticular chemicals.

Materials & Methods

Study Organisms & Location

Hawaiian spider specimens *T. brevignatha* and *T. stelarobusta* were hand collected from The Nature Conservancy of Hawaii's Lower Waikamoi Preserve in Maui, Hawaii on June 16, 2019. The undescribed dry habitat specialist species Crater Elongate Forest was hand collected near the Hōlua Campsite in Haleakalā National Park on June 15, 2019. And finally, the Californian species, *T. versicolor* was collected from Angelo Coast Range Reserve of the UC Natural Reserve System in Branscomb, California on June 25, 2019. Collected spiders were then given a paper towel saturated with water to allow for hydration. Climatic data for the Haleakala Crater and for Lower Waikamoi was obtained from Climate of Hawaii (Giambelluca et al. 2014) while the climatic data for Angelo Coast Range Reserve was obtained from the Angelo Headquarters' sensor data (Bode et al. 2018).

Desiccation Resistance Trials

To assess the physiological resistance to desiccation, desiccation resistance trials were conducted on *T. brevignatha*, *T. stelarobusta*, *T. versicolor* and Crater Elongate Forest spiders. Individual spiders were placed in 50mL falcon tubes with a single hole drilled into the caps. In each Falcon tube, 5000mg (±100mg) of Fischer Scientific clay drierite was placed at the bottom and covered with a cotton ball and a single bamboo stick was placed to allow a resting surface for the spiders. After spiders were given at least 24hrs to rehydrate after collection, they were then weighed and placed into individual Falcon tubes. The Falcon tubes were then placed into an insulated cooler box and maintained at a temperature range of 9°-15°C. Spiders were visually inspected approximately every 2-4hrs and checked for signs of life. Frequency of inspection was increased after a spider was determined to be near death. Following death, the time of death and the weight was recorded, and the spider was placed in a 5mL vial with a small amount of drierite and cotton placed at the bottom and stored at room temperature until further use. The trials continued until the spiders died or until around 197hrs had passed. If the spiders were not dead by that point, the trial was ended and the spiders were killed in a -80°C freezer and then stored in the same manner as the other spiders.

Metabolic Lipid Analysis

Total lipids were extracted from each sample using a modified Folch extraction (Folch, Lees and Sloane Stanley 1957). Samples were first homogenized using a glass pestle in a glass tube using a 2:1:0.9(v/v/v) mixture of chloroform:methanol:water. An internal standard of cholesterol was added, then the samples were vortexed for one minute, then incubated for ten minutes at room temperature, and centrifuged at 500 g for 10 minutes to separate organic and inorganic phases following Treidel et al 2021. The layers were separated, then the procedure was repeated again to insure minimal loss of organic layer. Samples were then dried and resuspended in chloroform.

Whole body lipid extract was then used to quantify triacylglycerides via thin-layer chromatography and flame ionization detection (TLF-FID) using an Iatroscan MK-6s TLC-FID analyzer (Shell-USA) following a protocol outlined in Williams et al 2011. Inorganic phase from the whole-body Folch extraction was then concentrated using a heated vacuum centrifuge (Get

specific model), then reconstituted in 0.01% Tween 20 solution. Absorbance for all colorimetric assays were measured using a Synergy H1 plate Reader (BioTek).

Data were analyzed in R v4.1.2 (Team 2020). Triglyceride quantity between species was analyzed by first controlling for body size using carapace width (CW), a mass independent measure of size, and then comparing total values between species via ANOVA. Post hoc comparisons were done using a TukeyHSD test. Intraspecific mass survival comparisons were done by first calculating the time that it takes for 50% of each species to die (LT_{50}). Each individual was then assessed as above average or below, and this was used as the response variable in a logistic regression, with triglyceride as the independent variable and carapace width as a covariate.

Chemical Analysis of Spider Extracts

To assess the chemicals found on the cuticle of the spiders, full body extracts of *T. brevignatha*, *T. stelarobusta*, *T. versicolor* and Crater Elongate Forest spiders were analyzed by GC/MS (Agilent 7890A/5975C). Body extracts from 11 female *T. brevignatha*, 15 female *T. stelarobusta*, 12 female *T. versicolor* and 4 female Crater Elongate Forest spiders were analyzed. Spiders were first anesthetized in a -20°C freezer and then transferred into a 2mL glass vial containing 0.2mL of dichloromethane (DCM). The legs were folded so that the entire body was submerged in the solvent and the whole spider was extracted for 30 min before it was taken out and stored in 70% ethanol. The extracts were then stored at -20°C. All tools were washed and cleaned using acetone, ethanol, and DCM before and after each step of the extraction. Before running on the GC-MS, all extract samples were fully evaporated under a gentle stream of nitrogen gas and then 50uL of DCM mixed with an internal dodecane standard was added to the sample. The gas chromatograph was fitted with a fused silica capillary column (Agilent, DB-5MS, 30 m x 0.32 mm x 0.25 μm) with helium as the carrier gas. Extracts were analyzed in splitless mode, with a column oven temperature program of 50 °C for 5 min, increased by 5 °C min−1 to 320 °C. Injector and transfer line temperatures were maintained at 250 °C. GC-MS data was analyzed in OpenChrom (Wenig and Odermatt 2010) by detecting chromatogram peaks using the First Derivative Peak Detector feature at the highest threshold. Peak integration areas and linear retention indices were calculated using the Trapezoid Peak Integrator and Retention Index Calculator features respectively. All integrated peak areas were visually inspected and corrected by manual integration if necessary. The compound size was calculated based on the total number of carbons in the molecule while the GC retention index, a measure that is generally well correlated with molecular mass and to a much looser extent, melting point, was used as an additional proxy of a compounds potential waterproofing properties (Gibbs 2002; Gibbs and Rajpurohit 2010).

Results

Desiccation Resistance Trials

Overall, the climate at both Lower Waikamoi and the Haleakala Crater was more consistent than the highly variable climate at Angelo Coast Range Reserve. However, between the two sites in Hawaii, the Haleakala Crater was significantly colder and drier than the Lower Waikamoi site (Figure 1; SM 1).

The desiccation resistance trials yielded significant interspecific variation in survival times between the four species tested. During the entire duration of the trials, only one out of the eight Crater Elongate Forest spiders died while all the *T. brevignatha, T. stelarobusta,* and all but one of the *T. versicolor* spiders died. Of the species that had all the individuals die during the duration of the experiment, on average individuals of *T. versicolor* survived the longest (*T. versicolor*: mean = 119.799 hrs, sd = 27.597) while *T. brevignatha* and *T. stelarobusta* had very comparable survival times (*T. brevignatha*: mean = 83.529 hrs, sd = 30.591; *T. stelarobusta*: mean = 83.996 hrs, sd = 26.635) (Figure 2; SM 2).

The initial mass of the Californian *T. versicolor* spiders was on average significantly heavier than individuals of the three Hawaiian *Tetragnatha* species (*T. versicolor*: mean = 41mg, sd = 15.483) (Figure 3; SM 3). Within the Hawaiian species, *T. stelarobusta* spiders trended slightly heavier than *T. brevignatha* and Crater Elongate Forest spiders, but this was not statistically significant (*T. stelarobusta*: mean = 29.934 mg, sd = 8.581; *T. brevignatha*: mean = 21.2 mg, sd = 5.185; Crater Elongate Forest: mean = 21.556 mg, sd = 8.218) (Figure 3; SM 3). Similar results were seen for body size where *T. versicolor* spiders had the largest body size (Figure 3, Figure 4, SM 3; SM 4; SM 5). Amongst the Hawaiian species, body size significantly differed depending on the specific measurement being made and the direction of difference varied accordingly (Figure 3, Figure 4, SM 3; SM 4; SM 5). Overall, *T. stelarobusta* trended larger than Crater Elongate Forest and *T. brevignatha* spiders but differences were not statistically significant much of the time (Figure 3, Figure 4, SM 3; SM 4; SM 5). The initial mass and body size of each species is tightly and positively correlated (Figure 3).

The average percentage of mass lost during the desiccation trials (whether at the point the spider died or at the end of the trial duration) did not statistically differ between species with *T. brevignatha* and *T. stelarobusta* spiders losing around 25.770% ± 8.467 and 30.410% ± 6.734, respectively, while Crater Elongate Forest and *T. versicolor* spiders lost a little less, around 23.331% ± 6.248 and 25.408% ± 7.337, respectively (Figure 5; SM 6). However, when controlled for the time the spiders were exposed to the trial (so the time until their death or in the Crater Elongate Forest spider's case, the time until the end of the trial) the average percent of mass lost (so the rate of mass loss) differed significantly between species with Crater Elongate Forest spiders losing significantly less mass than both *T. brevignatha* and *T. stelarobusta* spiders (Crater Elongate Forest: mean = 0.118, sd = 0.032; *T. brevignatha*: mean = 0.348, sd = 0.193; *T. stelarobusta*: mean = 0.395, sd = 0.153) (Figure 5; SM 6). This was also the case with the Californian *T. versicolor* spiders, which lost significantly less mass compared to *T. stelarobusta* spiders (*T. versicolor*: mean = 0.227, sd = 0.1) (Figure 5; SM 6). There was no statistically significant difference in the rate of mass loss between *T. brevignatha* and *T. stelarobusta* spiders as well as between Crater Elongate Forest and *T. versicolor* spiders (Figure 5; SM 6).

Metabolic Lipid Analysis

Mass-specific triglyceride abundance did differ between species ($F_{3,46}$= 3.68, p=0.0186) (Figure 6), with a post-hoc analysis revealing that *T. stelarobusta* had significantly higher triglyceride stores than both *T. brevignatha* (p = 0.0217) and *T. versicolor* (p = 0.0430). Crater Elongate Forest had intermediate triglyceride stores and did not differ from any other species. Intra-specific individual-based comparisons of triglyceride content found that when corrected for body size (carapace width), individuals with higher triglyceride stores were more likely to be in the upper 50% of survival duration within a species (z = 2.003, p= 0.045) (Figure 6B).

Chemical Analysis of Spider Extracts

The chemicals found on the cuticle of the *Tetragnatha* spiders analyzed in this study consisted of a mixture of methyl ethers, cuticular hydrocarbons, and unidentified compounds that were categorized as "other" (SM 7). Methyl ethers composed the majority of the chemical profile and comprised about 80.73% of a spider's chemical profile while the unidentifiable compounds comprised about 16.476% of the profile and cuticular hydrocarbons about 2.794% of the profile (Figure 7). The relative abundance of methyl ethers differed significantly between species with *T. brevignatha* having a significantly higher relative abundance of methyl ethers compared to *T. stelarobusta* and *T. versicolor* (*T. brevignatha*: mean = 89.086, sd = 4.323; *T. stelarobusta*: mean = 74.74, sd = 9.2; *T. versicolor*: mean = 78.970, sd = 6.265) (Figure 7; SM 8). Although Crater Elongate Forest spiders also had higher relative abundances of methyl ethers compared to *T. stelarobusta* and *T. versicolor* it was not statistically significant (Crater Elongate Forest: mean = 85.497, sd = 10.115) (Figure 7; SM 8). The relative abundances of cuticular hydrocarbons also differed between species. Both *T. versicolor* and Crater Elongate Forest spiders had significantly higher relative abundances of cuticular hydrocarbons than *T. brevignatha* and *T. stelarobusta* (*T. versicolor*: mean = 5.705, sd = 2.659; Crater Elongate Forest: mean = 4.542, sd = 5.317; *T. brevignatha*: mean = 1.114, sd = 0.635; *T. stelarobusta*: mean = 1.23, sd = 1.081) (Figure 7; SM 8). Specifically, both *T. versicolor* and Crater Elongate Forest spiders had significantly higher relative abundances of methyl-branched alkanes than *T. brevignatha* and *T. stelarobusta* (*T. versicolor*: mean = 3.704, sd = 2.036; Crater Elongate Forest: mean = 3.91, sd = 4.822; *T. brevignatha*: mean = 0.548, sd = 0.350; *T. stelarobusta*: mean = 0.673, sd = 0.709) (Figure 8; SM 9), while not statistically significant, *T. brevignatha* and *T. stelarobusta* had slightly higher relative abundances of alkenes compared to *T. versicolor* and Crater Elongate Forest spiders (*T. versicolor*: mean = 0.281, sd = 0.653; Crater Elongate Forest: mean = 0.253, sd = 0.315; *T. brevignatha*: mean = 0.506, sd = 0.336; *T. stelarobusta*: mean = 0.437, sd = 0.429) (Figure 8; SM 9) and *T. versicolor* had slightly higher relative abundances of straight chain alkanes compared to the other spiders (*T. versicolor*: mean = 0.228, sd = 0.188; Crater Elongate Forest: mean = 0.379, sd = 0.673; *T. brevignatha*: mean = 0.0533, sd = 0.0758; *T. stelarobusta*: mean = 0.12, sd = 0.167) (Figure 8; SM 9). Within the methyl-branched alkanes, mono-methyl alkanes with the methyl branch on the 4th carbon was the most prevalent compound found in all the species analyzed. Only *T. versicolor* and Crater Elongate Forest spiders had methyl-branched alkanes with differing branching patterns. Finally, for the remaining "other" compounds, *T. stelarobusta* had significantly higher relative abundances of these compounds compared to all the other spiders (*T. stelarobusta*: mean = 24.03, sd = 8.953) but the remaining spiders did not differ significantly (*T. versicolor*: mean = 15.325, sd = 4.706; Crater Elongate Forest: mean = 9.961, sd = 5.005; *T. brevignatha*: mean = 9.8, sd = 4.189) (Figure 7; SM 8).

As for compound size and retention index, there were significant interspecific differences in both measures for the cuticular hydrocarbons and methyl ethers found on the spiders. On average, *T. brevignatha* and *T. stelarobusta* had larger methyl ethers with significantly higher retention index (Size: *T. brevignatha*: mean = , sd = ; *T. stelarobusta*: mean = , sd = ; Retention Index: *T. brevignatha*: mean = 3362.676, sd = 68.241; *T. stelarobusta*: mean = 3410.463, sd = 97.964) than *T. versicolor* and Crater Elongate Forest (Size: *T. versicolor*: mean = , sd = ; Crater Elongate Forest: mean = , sd = ; Retention Index: *T. versicolor*: mean = 3213.987, sd = 46.768; Crater Elongate Forest: mean = 3197.786, sd = 73.922) (Figure 9; SM 10; SM 11; SM 12), while the opposite is true for cuticular hydrocarbons where *T. versicolor* and Crater Elongate Forest had larger sized cuticular hydrocarbons with significantly high retention index (Size: *T. versicolor*: mean = , sd = ; Crater Elongate Forest: mean = , sd = ; Retention Index: *T. versicolor*: mean = 3085.845, sd = 95.302; Crater Elongate Forest: mean = 2955.354, sd = 73.115) than *T. brevignatha* and *T. stelarobusta* (Size: *T. brevignatha*: mean = , sd = ; *T. stelarobusta*: mean = , sd = ; Retention Index: *T. brevignatha*: mean = 2528.955, sd = 54.923; *T. stelarobusta*: mean = 2620.234, sd = 90.804) (Figure 9; SM 10; SM 11; SM 12). A similar trend was found when the cuticular hydrocarbons were separated into the three categories of alkanes, methyl-alkanes, and alkenes. The alkanes, methyl-alkanes, and alkenes found on *T. versicolor* and Crater Elongate Forest spiders were consistently much larger in size with a significantly higher retention index value than those found on *T. brevignatha* and *T. stelarobusta* (Figure 8; SM 13; SM 14; SM 15). Although the compound size was not calculated for the unidentified compounds found on the spiders, the retention index was calculated and showed that *T. versicolor* and Crater Elongate Forest had identified compounds with higher retention index (*T. versicolor*: mean = 3116.084, sd = 74.68; Crater Elongate Forest: mean = 3093.477, sd = 98.745) than those found on *T. brevignatha* and *T. stelarobusta* (*T. brevignatha*: mean = 2937.933, sd = 98.491; *T. stelarobusta*: mean = 2963.914, sd = 100.127) (SM 10; SM 12).

Discussion

Our study shows that there are interspecific differences in the desiccation resistance, metabolic lipid, and cuticular chemical of the four *Tetragnatha* species. In the desiccation experiments, spiders from arid environments had a lower rate of mass loss and survived longer than those from wetter environments. The cuticular chemicals of these longer surviving species consisted of overall more cuticular hydrocarbons, as well as cuticular hydrocarbons of a larger size (total carbon) and higher retention index than those from wetter environments. However, this was not the case for methyl ethers nor metabolic lipids and the amounts of these components did not correspond with desiccation resistance. Spiders that survived longer had smaller methyl ethers with lower retention index – the direct opposite trend from cuticular hydrocarbons.

Desiccation Resistance Between Habitats

The Haleakala crater is consistently and significantly colder and drier throughout the year than the nearby wet forest habitats (Figure 1). Not surprisingly, it also experiences significantly more solar radiation throughout the year than the wet forest. Consequently, it was not surprising that the “Crater Elongate Forest” spiders that inhabit this cold, dry, and light intensive environment

survived better and had significantly lower rates of mass loss than their closely related wet forest counterparts, *T. brevignatha* and *T. stelarobusta*. What was surprising was that they were so desiccation resistant that only 2 out of the 9 individuals died during the entire duration of the desiccation resistance trials while all the individuals of the wet forest species had perished by the same time. Even when compared to *T. versicolor*, a species found in the relatively hot, dry, and light intensive California climate, they outperformed significantly. Broad-scale, multi-species studies conducted on beetles have shown strong correlations between habitat aridity and desiccation resistance (Chown and Nicolson 2004). The *Tetragnatha* spiders show a similar trend with the dry habitat specialist, Crater Elongate Forest, having the highest desiccation resistance followed by the Californian *T. versicolor* from the second driest habitat. Although Haleakala Crater experiences the lowest annual air temperatures compared to the other locations, it is possible that the combination of the low air temperatures and the high solar radiation encountered at the site actually significantly increases the experienced body temperatures of the spiders that live there. Studies conducted on grasshoppers have shown that the actual body temperature of individual grasshoppers varied from the surrounding air temperature and showed to increase the most, as much as 12°C, when individuals were exposed to low air temperatures and high light intensities (Pepper and Hastings 1952). Therefore, it is possible that the spiders that inhabit the Haleakala Crater experience higher body temperatures than what is expected from air temperature measures alone, necessitating more effective measures against desiccation than habitats with higher air temperatures.

Desiccation Resistance Between Lifestyles

Tetragnatha brevignatha and *T. stelarobusta* can be found in the same wet forest habitat of Lower Waikamoi but have very different life history strategies. *Tetragnatha brevignatha* is a non-web building spider that lives a cursorial lifestyle while *T. stelarobusta*, like the Crater Elongate Forest and *T. versicolor* spiders, is a web-building spider. Both individuals of *T. brevignatha* and *T. stelarobusta* died at a similar rate in the desiccation resistance trials and even had very comparable rates of mass loss (Figure 2; Figure 5). It seems that the minor size difference between *T. brevignatha* and *T. stelarobusta* (Figure 3; Figure 4) was not large enough to confer any differences in water loss between the two species as is seen in other insects and spiders with significant size differences (Schmitz 2016; Kühsel et al. 2017). It also seems that the lifestyle differences found between *T. brevignatha* and *T. stelarobusta* do not affect desiccation resistance and water loss. Although it has been shown that cursorial spiders have higher metabolic rates, and therefore respiratory water loss than web-building spiders, it is possible that the cursorial *T. brevignatha* are not as active compared to other cursorial spiders that stalk and attack their prey (Schmitz 2016). *Tetragnatha brevignatha* have been seen more readily staying in one location with their legs open seemingly waiting for prey, then stalking and attacking prey (personal observations). Another possibility is that respiratory water loss does not play a large role in the overall process of water loss in spiders and evaporative water loss from cuticular surfaces may be more impactful (Vollmer and MacMahon 1974). Spiders are notorious for having incredibly low respiratory rates compared to other arthropods of comparable size and are known to have a high anaerobic capacity (Schmitz 2016; Hsia et al. 2014). Spiders can partition between aerobic and anaerobic metabolism during medium level activities such as web-building and even ambush-style spiders were found relying on anaerobic respiration for short bursts of high-level activity (Schmitz 2016). Respiratory water loss does indeed happen in spiders, but it

was found to be much less than in insects (Davies and Edney 1952). Furthermore, in some insects it has been found that respiratory water loss constitutes a significant fraction of the total water loss only when cuticular transpiration was drastically reduced, thereby indicating that naturally, a large portion of the water loss comes from cuticular transpiration rather than respiration (Chown 2002; Gibbs and Rajpurohit 2010; Quinlan and Gibbs 2006). Thus, it is possible that respiratory water loss plays an even smaller role in the overall process of water loss in spiders than has been shown in insects.

Desiccation Resistance & Metabolic Lipids

Metabolic water can be an important source of water in many organisms where extra water can be stored in the form of glycogens and metabolic lipids such as triglycerides (Gibbs, Chippindale, and Rose 1997). The amount of triglycerides varied interspecifically in the spiders, but the relative amounts did not correspond with the desiccation trends seen in the spiders. *Tetragnatha stelarobusta*, one of the least desiccation resistant spiders, had significantly higher triglyceride stores than the other spiders while *T. versicolor*, the second desiccation resistant spider, had the lowest stores and Crater Elongate Forest, the most desiccation resistant spider, had medium amounts. While there were differences in triglyceride stores and desiccation survival times at the individual level, it seems that there is not a strong species-specific association of metabolic lipid stores and desiccation resistance. Studies conducted on *Drosophila* have found that the relative amount of metabolic lipids such as triglycerides vary greatly in lines selected for desiccation resistance compared to control lines (Ko et al. 2019) while a study conducted on an extremely desiccation tolerant Antarctic midge found no changes in the amount of triglycerides after exposure to desiccation (Teets et al. 2012). However, many of these studies found that glycogen seems to play a more significant role in desiccation resistance and find that desiccation resistant insects have more glycogen stores (Ko et al. 2019; Teets et al. 2012). Unfortunately, the glycogen levels in the spiders for this study were so low that they were not clearly detected and thus a clear conclusion could not be drawn about the role glycogen plays in the desiccation resistance of these spider (unpublished data).

Chemicals for Desiccation Resistance & Mate Choice

Previous studies of *Tetragnatha* spiders have shown that they use species-specific combinations of methyl ethers found on the silk of female spiders as mate recognition cues (Adams et al. 2021). These same methyl ethers were found in abundance on the cuticle of the spiders, yet these compounds did not correlate with the resulting desiccation resistance of the spiders (Figure 7). In many arthropods, the amount of cuticular lipids, especially hydrocarbons found on the cuticle of an individual are highly correlated with resisting desiccation and living in hotter, less humid environments (Chung and Carroll 2015; Buellesbach et al. 2018; Hadley, Ahearn and Howarth 1981). Artificial selection in *D. melanogaster* for more desiccation resistant populations led to an increase in the amount of cuticular hydrocarbons found in these populations (Ferveur et al. 2018), while exposing scorpions to desiccation stress resulted in a significant increase in the total amount of cuticular hydrocarbons extracted from these individuals (Gefen et al. 2015). However, in our study, we did not see this pattern of increase in the relative abundance of methyl ethers on the most desiccation resistance spiders. In contrast, like what is found in insects, the most desiccation resistant species, Crater Elongate Forest and *T. versicolor,* had a significantly higher

proportion of cuticular hydrocarbons in their profile than did the two least desiccation resistant species, *T. brevignatha* and *T. stelarobusta* (Figure 7).

The melting temperature of a compound has been shown to have a strong influence on the permeability of water through the cuticle and thereby the desiccation resistance of an organism (Gibbs and Rajpurohit 2010). Compounds with higher melting temperatures are less permeable and are better suited to prevent water loss from the cuticles (Gibbs and Rajpurohit 2010). Saturated, straight-chained cuticular hydrocarbons have the highest melting temperatures, and the melting temperature decreases with the addition of a methyl branch, double bond, and decrease in chain length (compound size) (Gibbs and Rajpurohit 2010). The compound size of the methyl ethers in our spiders showed the opposite trend to what would be expected of compounds best suited for desiccation resistance. The most desiccation resistance species, Crater Elongate Forest and *T. versicolor* had the smallest methyl ethers while the least desiccation resistant species *T. brevignatha* and *T. stelarobusta* had the largest methyl ethers (Figure 9). The same pattern was also observed in the retention index of the methyl ethers, a measure that correlates well with molecular size (Gibbs and Rajpurohit 2010) (SM 10). However, again in contrast, the size and retention index of the cuticular hydrocarbons showed the opposite trend with the two most desiccation resistant species, Crater Elongate Forest and *T. versicolor* having the largest cuticular hydrocarbons while the two least desiccation resistant species *T. brevignatha* and *T. stelarobusta* having the smallest cuticular hydrocarbons (Figure 9). Even when the cuticular hydrocarbons were separated into the three respective categories of alkanes, methyl-alkanes, and alkenes, the same results were obtained with the two most desiccation resistant species having the largest compounds in each category (Figure 8). There is ample evidence that larger hydrocarbons with a longer-chain length contributes greatly to an increased desiccation resistance in arthropods (Chung and Carroll 2015; Buellesbach et al. 2018; Ferveur et al. 2018; Gefen et al. 2015; Xu et al. 2018; Blomquist and Ginzel 2021) and it seems that the cuticular hydrocarbons found on the spiders of this study exhibit the same trend. As for the effects of methyl branches and double bonds on the desiccation resistance of arthropods, evidence is less straightforward, yet it is suggested that arthropods in warmer, drier climates produce more saturated cuticular hydrocarbons (straight-chained compounds including methyl-alkanes) while those found in cooler, wetter climates have a higher proportion of unsaturated compounds (compounds with double bonds) and fewer methyl-alkanes (Chung et al. 2014; Chung and Carroll 2015; Gefen et al. 2015; Xu et al. 2018; Blomquist and Ginzel 2021; Menzel, Blaimer and Schmitt 2017). The same trend was found in the *Tetragnatha* spiders where the two most desiccation resistant species, Crater Elongate Forest and *T. versicolor* had a higher proportion of methyl-alkanes and a lower proportion of alkenes compared to the two least desiccation resistant species *T. brevignatha* and *T. stelarobusta* (Figure 8).

In agreement with previous findings, the most abundant methyl ethers found on the cuticle of the spiders in this study matched the species-specific methyl ethers found on the silk of the spiders from previous studies (Adams et al. 2021). As such, the most abundant methyl ethers on the cuticle were highly species-specific with very little overlap between species. In contrast, the cuticular hydrocarbons found on the spiders overlapped more between species with the same compound frequently found across all four species (for example, 4-methylhexacosane, 4-methyloctacosane, and 4-methyltriacontane). Together with the relative abundance and compound size results, it seems that methyl ethers do not play a large role in the desiccation

resistance of *Tetragnatha* spiders and instead, as seen in many other arthropods, cuticular hydrocarbons play a more important role. Cuticular hydrocarbons are often the most hydrophobic lipid in the mixture of cuticular lipids found from arthropods and have been suggested to provide the best barrier to water loss (Gibbs and Rajpurohit 2010). However, unlike many insects where cuticular hydrocarbons serve as both mate recognition cues and chemicals used for desiccation resistance, the two roles of mate recognition and desiccation resistance seem to be represented by two different classes of compounds in these spiders. Although this means that the methyl ether compounds most likely do not function as magic traits that help accelerate the process of speciation under divergent selective regimes, it also means that there is most likely little opposing conflict between selection for mate recognition and desiccation resistance. In insects where cuticular hydrocarbons are involved in both mate recognition and desiccation resistance, it is proposed that there is opposing conflict in producing compounds best suited for communication (short chained, unsaturated and methylated compounds) and those best suited for desiccation resistance (long chained saturated compounds without methyl branches) (Chung and Carroll 2015; Montooth and Gibbs 2003). The separation of these two roles of mate recognition and desiccation resistance in spiders, may allow the compounds used in mate recognition more flexibility to diverge freely. Nevertheless, this study found that mate recognition and desiccation resistance are performed by different classes of chemicals and suggests that accelerated reproductive isolation is attained without the aid of the powerful isolating force of magic traits.

Conclusion

The study highlights the physiology of the closely related Hawaiian *Tetragnatha* spiders that inhabit extremely divergent wet and dry habitats and the role cuticular lipids play in the desiccation resistance of these spiders. As expected, species found in drier environments survived much longer in the desiccation assays than their closely related wet habitat counterparts, while contradictory to what was expected, the evolutionary lifestyle differences of the spiders (web building vs cursorial) seem to not play a large role in desiccation resistance. Furthermore, unlike what is found in many insects, the compounds used for mate recognition were not correlated with the desiccation resistance properties of the spiders. In turn, the two roles of mate recognition and desiccation resistance appear to be handled by separate compound types – methyl ethers and cuticular hydrocarbons respectively. These findings provide key insights into the role cuticular lipids play in the rapid divergence of species experiencing ecologically driven divergent selection.

Figures & Tables

Fig 1 – Monthly average air temperature, relative humidity, and the solar radiation measured at the three study sites of Haleakala Crater (Maui), Lower Waikamoi (Maui), and Angelo Coast Range Reserve (California).

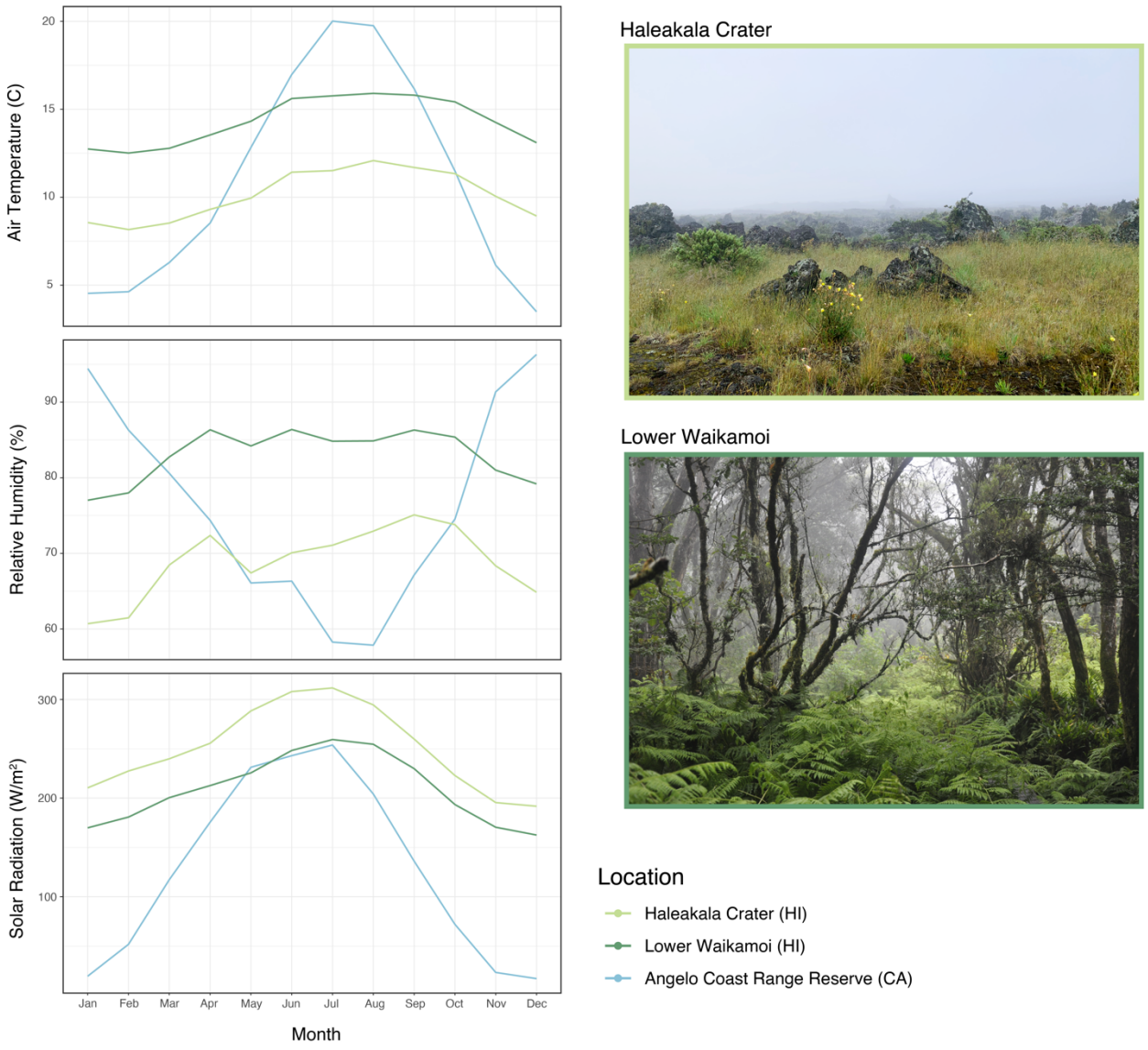


Fig 2 – Results of the desiccation resistance assays for the four *Tetragnatha* species. The LT50 for each species were: *T. brevignatha* = 78.512 hrs, Crater Elongate Forest = not applicable, *T. stelarobusta* = 83.480 hrs, *T. versicolor* = 122.227 hrs.

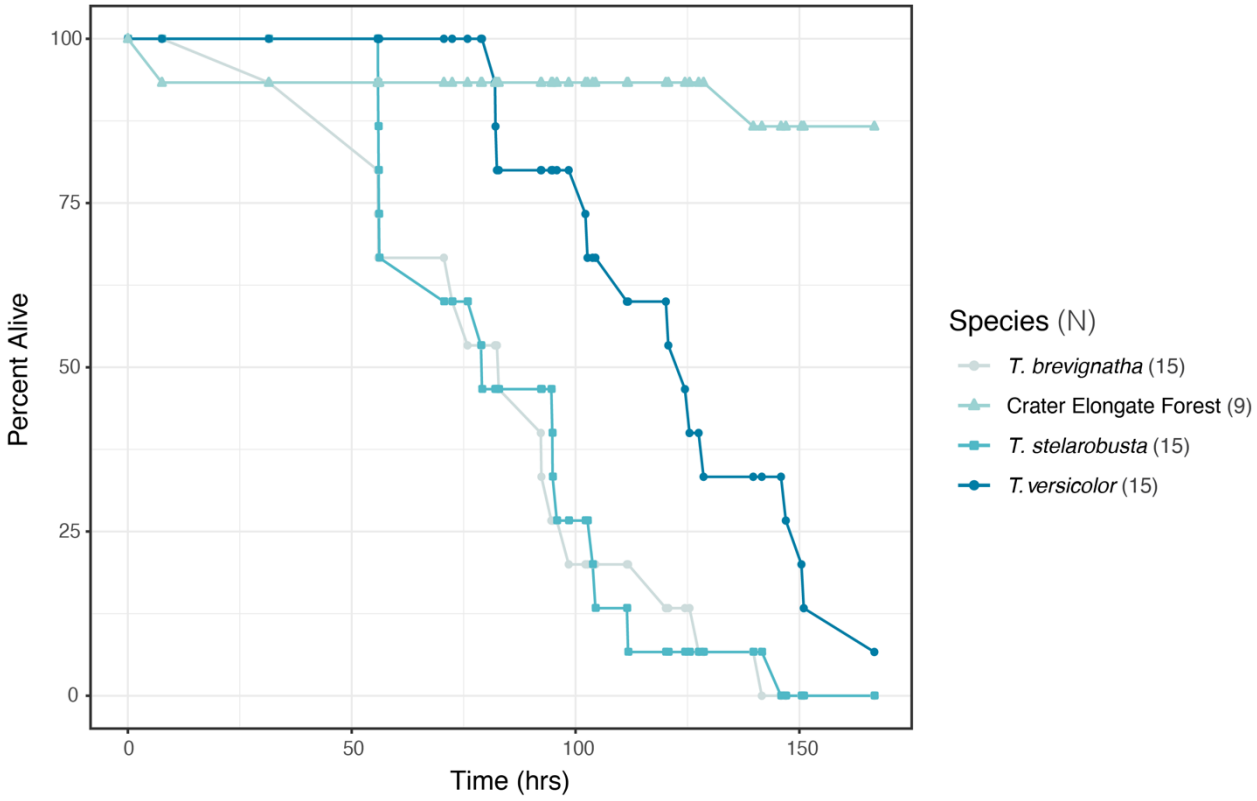


Fig 3 – The initial mass and body size measurements of each species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

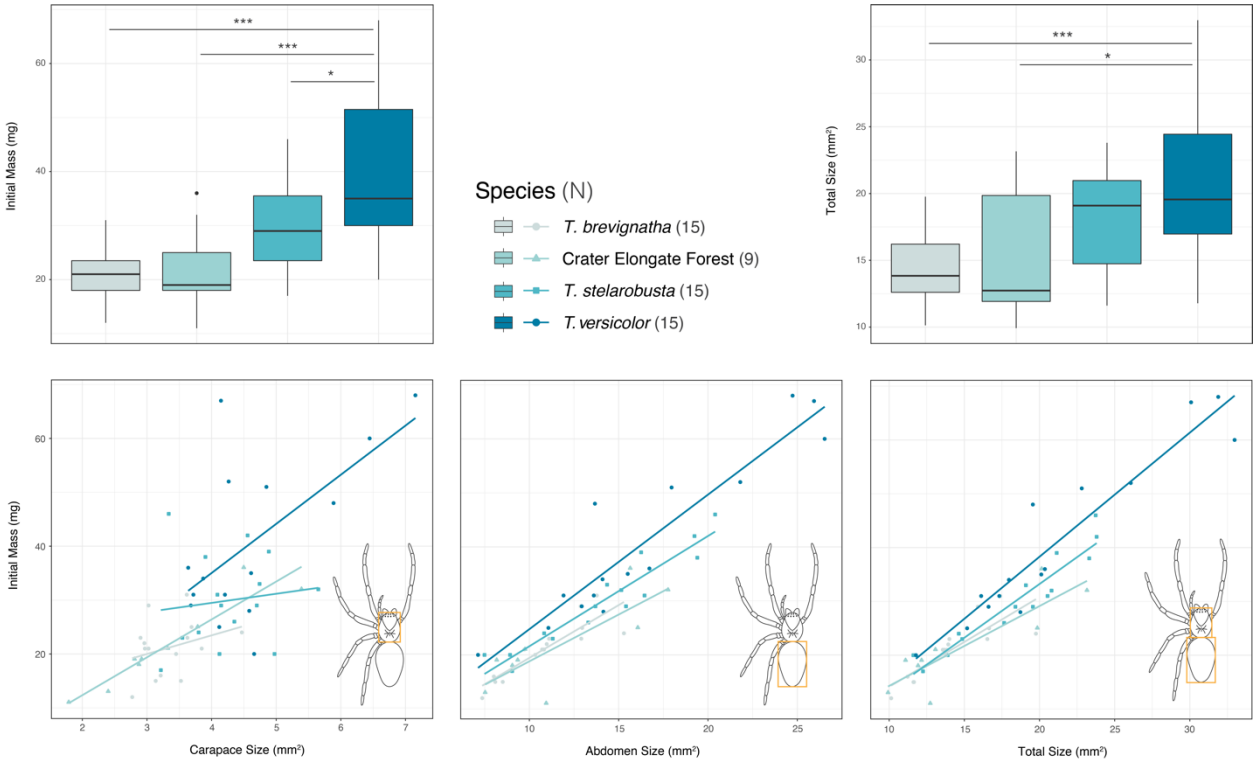


Fig 4 – The specific body measurements made of each species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

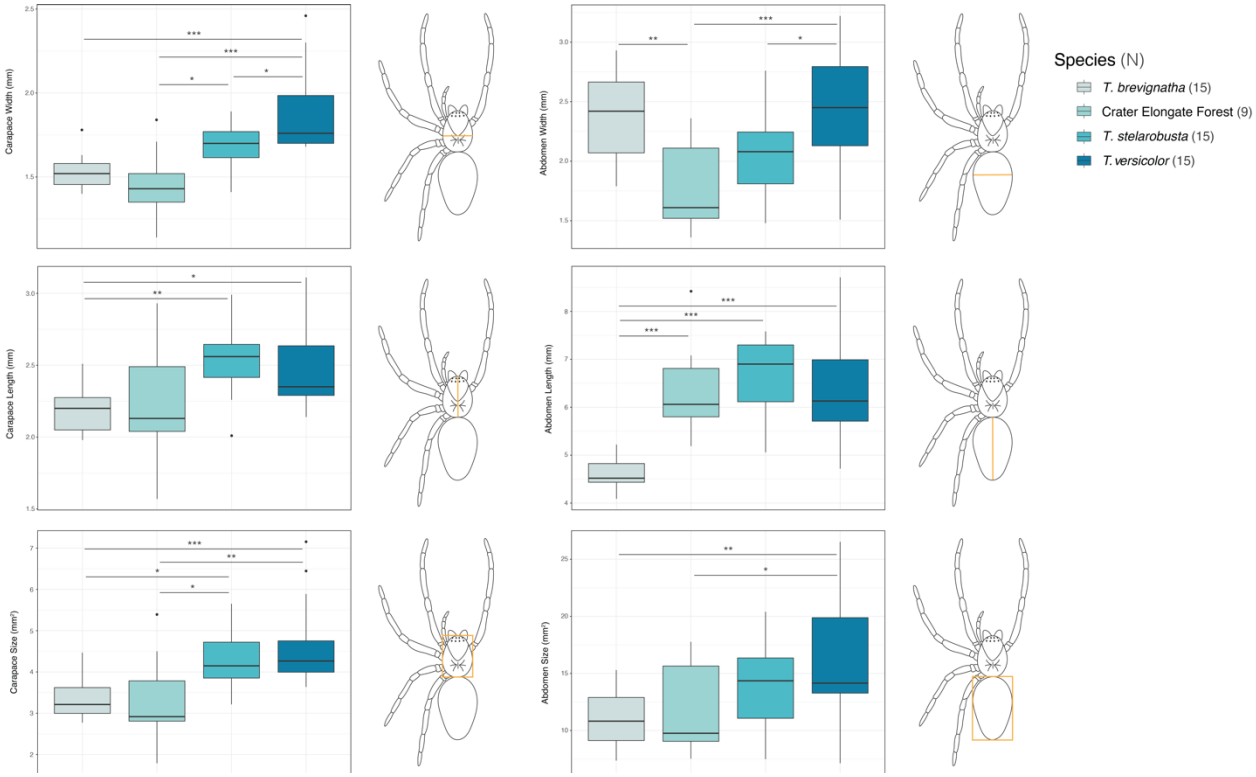


Fig 5 – Percent of mass lost during the desiccation resistance trials and the rate of water lost in each species. Data from Crater Elongate Forest spiders are only from those that were still alive at the end of the experiment. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

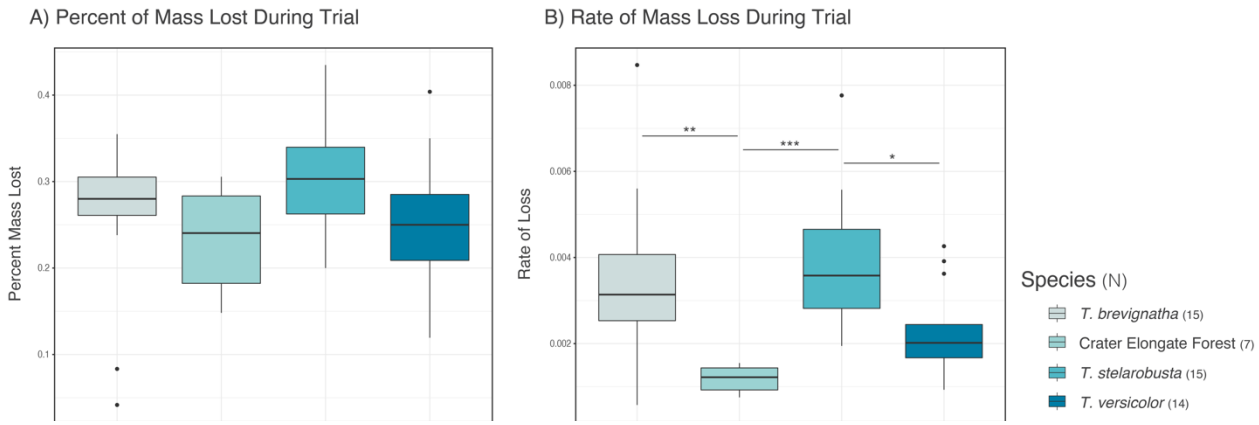


Fig 6 – The amount of metabolic lipids found in each species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

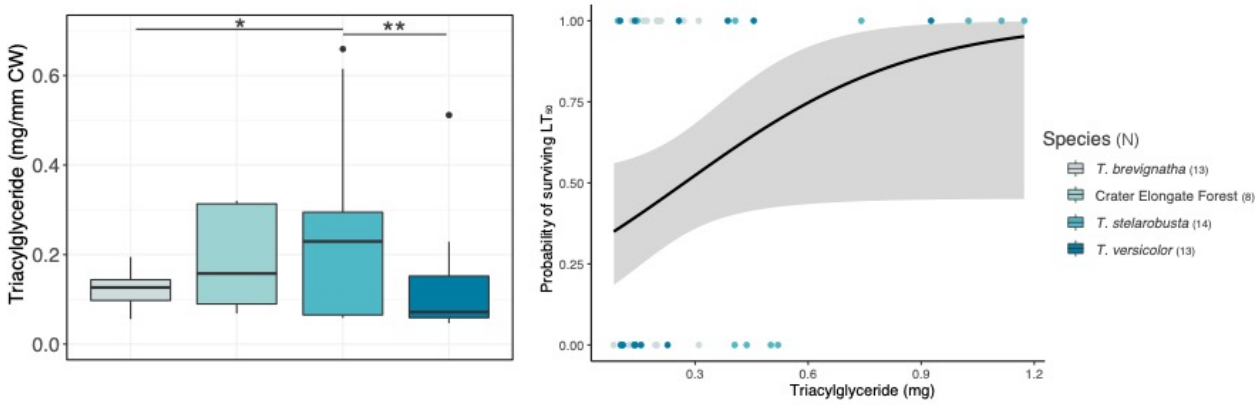


Fig 7 – Relative proportions of methyl ethers (MEs), cuticular hydrocarbons (CHCs), and other unknown compounds found in the whole body extracts of each species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

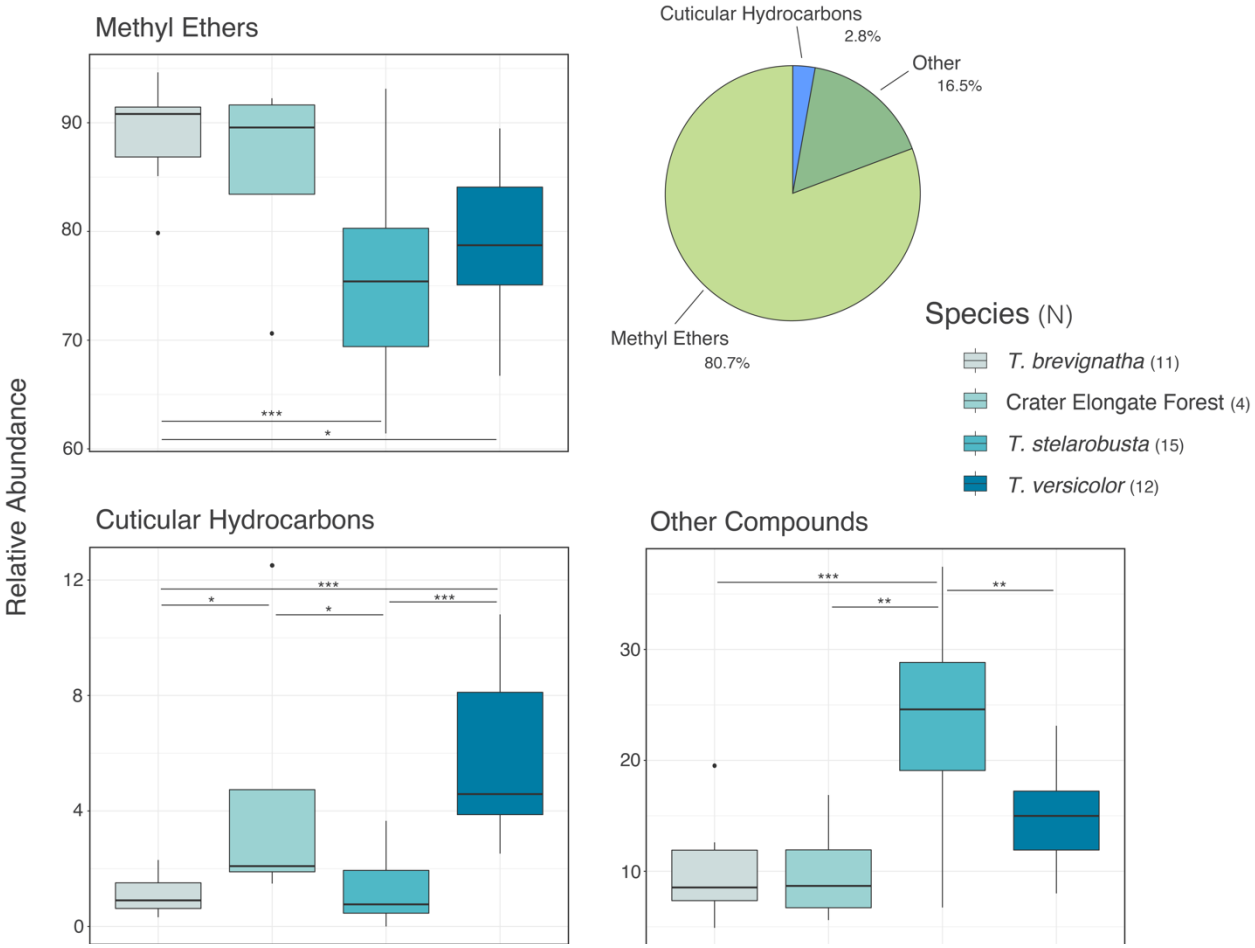


Fig 8 – Relative proportions and the compound size, measured as the total number of carbons in the molecule, of the various cuticular hydrocarbons (CHCs) found in the whole body extracts of each species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

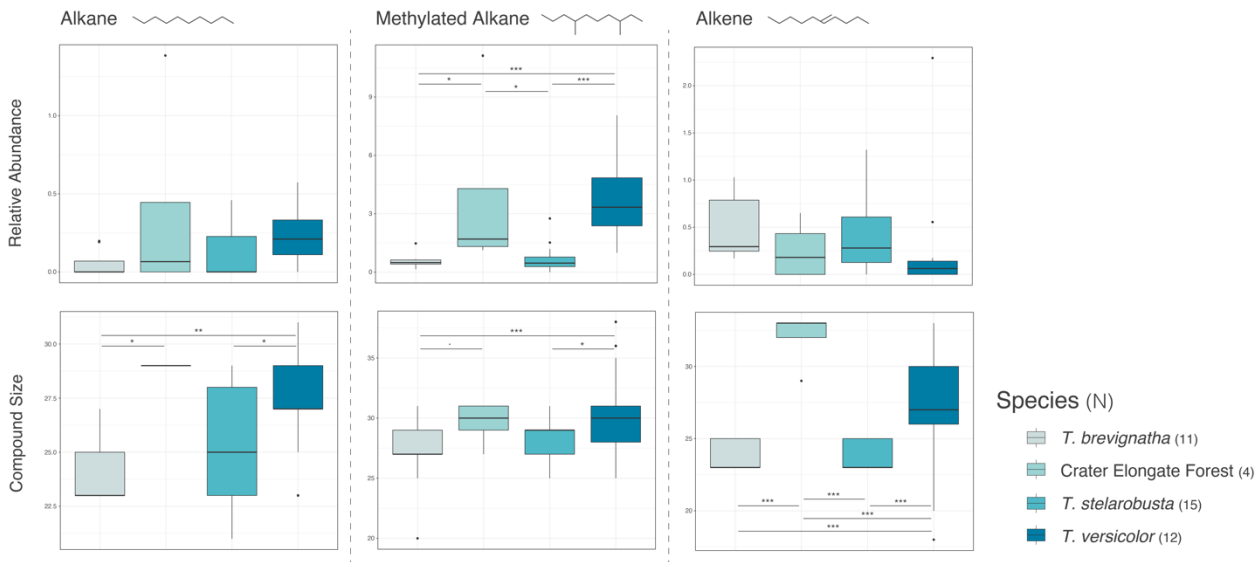
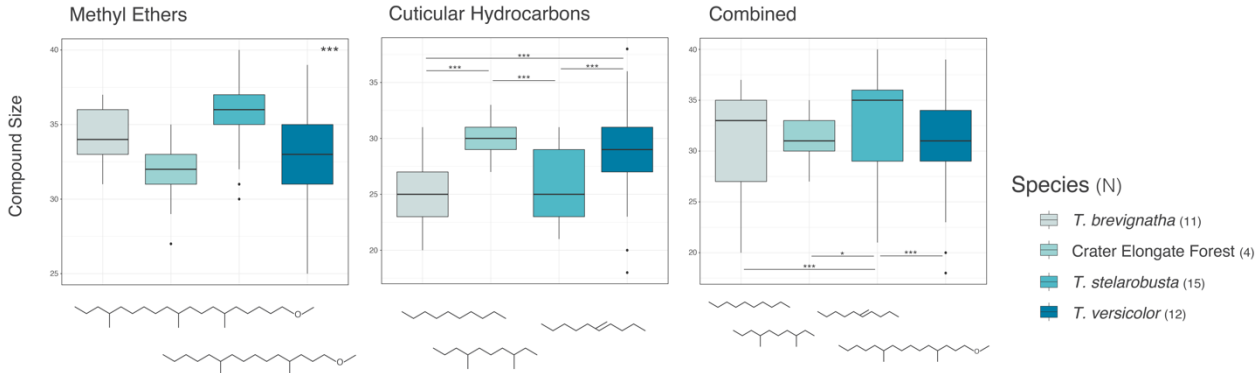


Fig 9 – Compound size, measured as the total number of carbons in the molecule, of the methyl ethers and cuticular hydrocarbons found in the species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).



Supplemental Material

SM 1 – The climatic averages of the three study sites.

	Air Temperature (°C)	**Relative Humidity (%)**	**Solar Radiation (W/m^2)**
Haleakala Crater (HI)	10.128 ± 1.425	68.886 ± 4.653	250.508 ± 42.662
Lower Waikamoi (HI)	14.311 ± 1.344	83.026 ± 3.394	209.025 ± 34.591
Angelo Coast Range Reserve (CA)	10.903 ± 6.139	76.127 ± 13.597	128.746 ± 91.437

SM 2 – TukeyHSD significance values for the time till death for all three species that died during the desiccation trials. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	p-values
Stella - Brevi	0.999
Vers - Brevi	0.004
Vers - Stella	0.004

SM 3 – TukeyHSD significance values for the initial mass and total size for all four species ran in the desiccation trials. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Initial Mass (p-values)	**Total Size (p-values)**
EF - Brevi	1.000	0.987
Stella - Brevi	0.107	0.086
Vers - Brevi	>0.001	>0.001
Stella - EF	0.229	0.302
Vers - EF	>0.001	0.012
Vers - Stella	0.025	0.353

SM 4 – TukeyHSD significance values for the carapace width, length, and size. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Carapace Width (p-values)	**Carapace Length (p-values)**	**Carapace Size (p-values)**
EF - Brevi	0.699	0.966	1.000
Stella - Brevi	0.747	0.006	0.019
Vers - Brevi	>0.001	0.034	>0.001
Stella - EF	0.011	0.067	0.040
Vers - EF	>0.001	0.214	0.002
Vers - Stella	0.022	0.915	0.580

SM 5 – TukeyHSD significance values for the abdomen width, length, and size. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Abdomen Width (p-values)	**Abdomen Length (p-values)**	**Abdomen Size (p-values)**
EF - Brevi	0.010	>0.001	0.980
Stella - Brevi	0.278	>0.001	0.175
Vers - Brevi	0.806	>0.001	0.003
Stella - EF	0.336	0.845	0.501
Vers - EF	0.001	0.990	0.037
Vers - Stella	0.044	0.935	0.396

SM 6 – TukeyHSD significance values for the percent of mass lost and rate of loss for all four species ran in the desiccation trials. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Percent of Mass Lost (p-values)	**Rate of Loss (p-values)**
EF - Brevi	0.874	0.003
Stella - Brevi	0.323	0.800
Vers - Brevi	0.999	0.120
Stella - EF	0.140	>0.001
Vers - EF	0.920	0.325
Vers - Stella	0.274	0.014

SM 7 – CSV file with the abundance, relative abundance, and retention index information of the chemical compounds found on the cuticle of each specimen. The file can be found at: https://doi.org/10.6078/D1B414

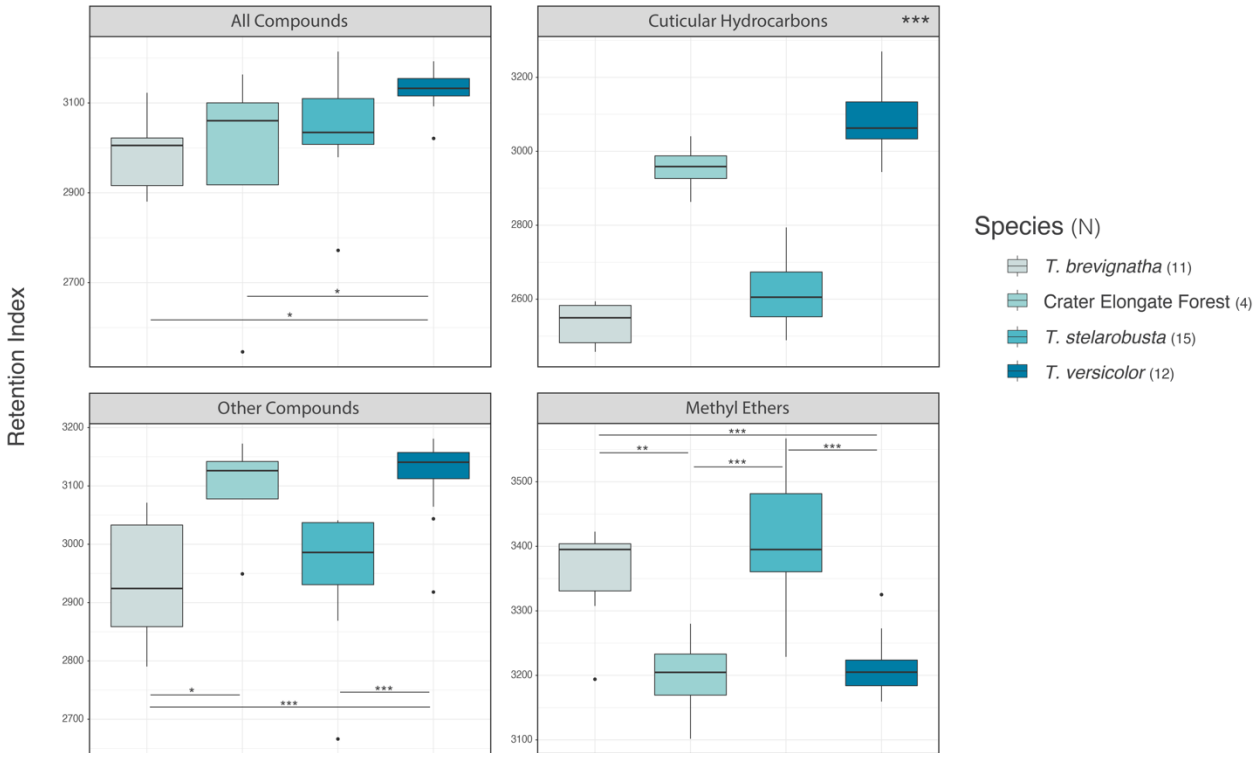
SM 8 – TukeyHSD significance values for the relative abundances of cuticular hydrocarbons, methyl ethers, and "other" compounds for all four species. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Cuticular Hydrocarbons (p-values)	**Methyl Ethers (p-values)**	**Other Compounds (p-values)**
EF - Brevi	0.050	0.842	1.000
Stella - Brevi	0.999	>0.001	>0.001
Vers - Brevi	>0.001	0.012	0.195
Stella - EF	0.050	0.066	0.002
Vers - EF	0.796	0.438	0.492
Vers - Stella	>0.001	0.468	0.007

SM 9 – TukeyHSD significance values for the relative abundances of alkanes, methyl-branched alkanes, and alkenes for all four species. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Alkanes (p-values)	**Methyl-Branched Alkanes (p-values)**	**Alkenes (p-values)**
EF - Brevi	0.111	0.014	0.801
Stella - Brevi	0.895	0.998	0.983
Vers - Brevi	0.316	>0.001	0.677
Stella - EF	0.239	0.015	0.902
Vers - EF	0.699	0.997	1.000
Vers - Stella	0.656	>0.001	0.835

SM 10 – The retention index of all the compounds, the cuticular hydrocarbons, the methyl ethers, and the unknown compounds categorized as “other” found in the cuticular extract of the four species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001). As indicated by the star on the top-right corner of the graph, all pair-wise comparisons between species for the cuticular hydrocarbons were statistically significant.



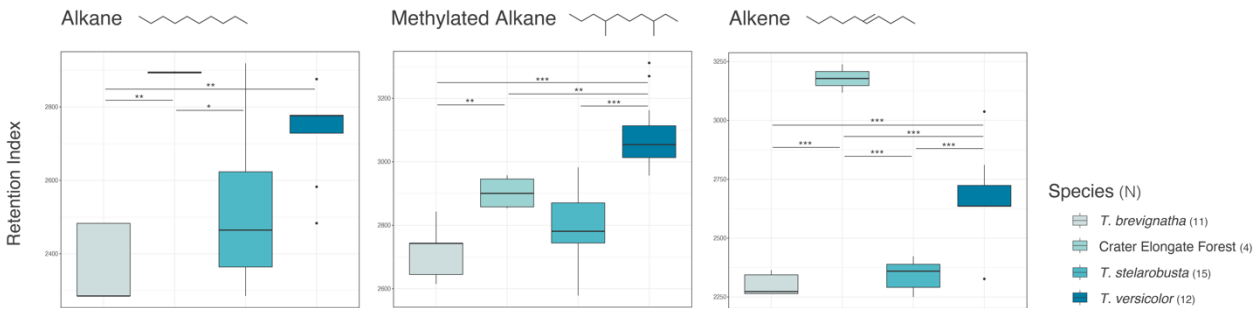
SM 11 – TukeyHSD significance values for the compound size measured as the total number of carbons of cuticular hydrocarbons, methyl ethers, and both combined for all four species. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Cuticular Hydrocarbons (p-values)	**Methyl Ethers (p-values)**	**Combined (p-values)**
EF - Brevi	>0.001	>0.001	0.995
Stella - Brevi	0.568	>0.001	>0.001
Vers - Brevi	>0.001	>0.001	0.860
Stella - EF	>0.001	>0.001	0.039
Vers - EF	0.557	0.015	0.993
Vers - Stella	>0.001	>0.001	>0.001

SM 12 – TukeyHSD significance values for the retention index of cuticular hydrocarbons, methyl ethers, and "other" compounds for all four species. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	All Compounds (p-values)	**Cuticular Hydrocarbons (p-values)**	**Methyl Ethers (p-values)**	**Other Compounds (p-values)**
EF - Brevi	0.981	>0.001	0.004	0.033
Stella - Brevi	0.446	0.045	0.403	0.895
Vers - Brevi	0.014	>0.001	>0.001	>0.001
Stella - EF	0.474	>0.001	>0.001	0.080
Vers - EF	0.047	0.045	0.983	0.974
Vers - Stella	0.234	>0.001	>0.001	>0.001

SM 13 – The retention index of the various cuticular hydrocarbons found in the cuticular extract of the four species. The degree of significance between comparisons are indicated by the star above each line (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).



SM 14 – TukeyHSD significance values for the compound size measured as the total number of carbons of alkanes, methyl-branched alkanes, and alkenes for all four species. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Alkanes (p-values)	**Methyl-Branched Alkanes (p-values)**	**Alkenes (p-values)**
EF - Brevi	0.039	0.055	>0.001
Stella - Brevi	0.676	0.392	0.976
Vers - Brevi	0.007	>0.001	>0.001
Stella - EF	0.120	0.465	>0.001
Vers - EF	0.743	0.985	0.001
Vers - Stella	0.026	0.015	>0.001

SM 15 – TukeyHSD significance values for the retention index of alkanes, methyl-branched alkanes, and alkenes for all four species. Abbreviations of the species names are as follows: Brevi = *T. brevignatha*, EF = Crater Elongate Forest, Stella = *T. stelarobusta*, Vers = *T. versicolor*. Statistically significant relationships are highlighted in grey.

	Alkanes (p-values)	**Methyl-Branched Alkanes (p-values)**	**Alkenes (p-values)**
EF - Brevi	0.003	0.005	>0.001
Stella - Brevi	0.343	0.076	0.870
Vers - Brevi	0.002	>0.001	>0.001
Stella - EF	0.034	0.241	>0.001
Vers - EF	0.536	0.010	>0.001
Vers - Stella	0.068	>0.001	>0.001

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