Host Specialization: Chemical Ecology of a Plant-Insect Herbivore System

A thesis
submitted in partial fulfillment of the requirements
of the degree of

Doctor of Philosophy

by

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20162002

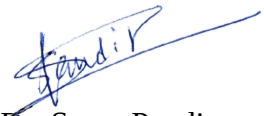
Indian Institute of Science Education and Research, Pune

2022

This thesis is dedicated to Achan, whose dream was to work in a forest, and Amma, whose dream was to pursue science.

Certificate

Certified that the work incorporated in the thesis entitled "Host Specialization: Chemical Ecology of a Plant-Insect Herbivore System" submitted by Ms. Gauri Binayak was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis previously submitted for the award of any degree or diploma from any other university or institution.



Dr. Sagar Pandit

Date: 31.07.2023

Declaration

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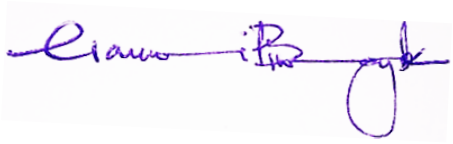

Gauri Binayak

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Date: 31.07.23

Acknowledgments

I express my heartfelt gratitude to my supervisor, Dr. Sagar Pandit, for his patience and guidance over the last six years. His energy and determination have been infectious during this journey, and I have thoroughly enjoyed the hours we spent brainstorming. I thank him for being so accepting of my ideas and the way I chose to express them.

I extend my thanks to my research advisory committee members, Prof. Sujata Bhargava and Prof. Aurnab Ghose, for their cooperation, availability, and patient review of my work progress.

Dr. Hemant Ghate's expertise in entomology has enriched this work, and I thank him for all the discussions amidst his busy schedule. I am incredibly grateful to Dr. Neelesh Dahanukar and Prof. Sutirth Dey for helping us with the multivariate statistics that allowed this project to expand. I must especially thank Dr. Dahanukar for spending countless hours patiently discussing the nuances of this work with me. I also thank Dr. Kesavan Subaharan and Vinay Kumar, our collaborators at ICAR National Bureau of Agricultural Insect Resources, Bangalore, for performing the electroantennography experiments.

I want to express my special thanks to Ashish Deshpande, Prajakta Ingale, and Sujay Paranjape, who have worked with me during various parts of this project. Their insights and lively discussions have made the process enjoyable and productive. I must also thank Greg DeSilva for his inputs with for odor imaging, and project interns Drashti Borad, Rutuja Ware, Vibhor Gaikwad, and Gargee Joshi for their inputs and cooperation.

My Ph.D. journey has relied heavily on the support of three colleagues, Rutwik Bardapurkar, Manish Kumar, and Rituparna Ghosh. Starting from building the lab from the ground-up, we have grown together, complemented each other's strengths, and learned to work as a team. More importantly, I have found the warmth of friendship and family with them. I will miss our free-flowing talks about science, life, and personal growth, and the pep talks we exchanged during struggles. I thank Rutwik, my regular tea break companion, for the space we held to bounce off ideas and hunches, even when they were difficult to articulate. We shared a rare bond where light-hearted and challenging conversations flowed equally comfortably, which undoubtedly contributed to my ability to think creatively. I want to thank Rituparna for all the moral support, reality checks and for teaching me GC-MS-FID techniques. I thank Manish for his utmost patience and sincerity in helping me troubleshoot and analyze metabolomics data. On several occasions, Surhud, Mahendra, Maruf, Rutwik, and Manish have selflessly taken time out from their schedules and accompanied me on field visits to collect plants and insects from the forests. I am deeply grateful to them for all the help. I could not have asked for better colleagues during my Ph.D.

Our field assistant, Mr. Ganesh Pawar, has been a pillar of support over these years. I thank him for maintaining the field and insectarium, providing so many home-cooked

meals, and always rushing to help whenever I needed it, even when it caused him inconvenience.

I am indebted to IISER Pune for providing me with the platform, fellowship, and world-class infrastructure to conduct my research. I thank CSIR for supporting me with the CSIR-NET JRF and SRF fellowships, which have been my major source of financial support. I also thank the Maharashtra State Biodiversity Board and Maharashtra Forest Department for providing us with permits to collect samples from the wild for this research.

My tenure in IISER was enriched by my friends, Himani Khurana, Aparna Sundaresan, Kaveri Vaidya, Shirsa Palit, Shridhar Hegde, and Soumya Bhattacharyya. Over the last six years away from home, living away from family has been made bearable and indeed enjoyable because of these wonderful people. I also want to thank Aswin for being such a calming presence and providing much-needed reality checks. Adheena, Saad, Nithin, Shyam, Thejus, and Alex have been a constant source of encouragement and sometimes, a much-needed reminder of a world outside the lab. I would not have reached the path of a Ph.D. without a young Ajay's support in my dreams, and I want to thank him for unknowingly bringing me to this journey. I am also thankful to Navaneeth for his faith in me.

My passion for science was born with and fostered by four teachers: Mrs. Erum, Mrs. Smitha VR, Mrs. Omibala, and Dr. Deepthy Alex. I am eternally grateful to these wonderful women for their teachings and patience with all my pestering questions. Without a doubt, they are the reason I fell in love with science and decided to pursue it. I must also mention my late botany professor Dr. Thomson Kuruvila and remember with gratitude his confidence in my capabilities that kept me going during tough times.

Lastly, I want to express my gratitude to my family. My mother, Geetha Binayak, dreamed of pursuing science, and my father, Binayak Shankar, dreamed of working in a forest. Their sacrifices towards my education have let me live a combination of their dreams. I am so fortunate to have them support my journey even when they didn't fully understand it. I want to thank my sister Ar. Aarati Binayak for being my constant in a world of variables, and my cousin Parvathy Gireesh, who gave me the courage to swim against the tide and pursue a career in what I love.

Contents

Synopsis

Title: Host Specialization: Chemical Ecology of a Plant-Insect Herbivore System

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Date of registration of Ph.D.: 1st August, 2018

Date of registration of Int. Ph.D.: 1st August, 2016

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Chapter 1: Introduction

Plant-insect herbivore systems encompass a large portion of terrestrial food webs. Insect herbivores often exhibit selectivity in their diet choices and stringently maintain their hostplant associations. Insects effect their hostplant location and identification mainly with the help of their highly specialized olfactory perceptions of plant odor cues. Plant odor is comprised of a volatile organic compounds (VOCs) mixture released into the headspace. Insects have evolved to perceive these volatiles as long-distance signals guiding to their hostplants. Apart from being cues used by insects for plant recognition, these volatile emissions have several ecological functions, such as attracting pollinators, mediating plant-plant communication, and mediating direct and indirect plant defense. Plant odor perception and hostplant identification in insects has been chiefly studied using a single model or crop systems. However, most natural habitats consist of complex vegetations. In several habitats, closely related plant species with similar chemical repertoires co-occur and add to the odorscape complexity. How foraging insect herbivores resolve such complex cue mixtures to identify their hostplant is poorly understood. To reveal the basis of such resolution, we studied a wild sympatric system from the Western Ghats: five plants of the genus *Ipomoea* (*I. batatas*, *I. carnea*, *I.*

elliptica, *I. triloba*, and *I. parasitica*) and four specialist *Chiridopsis* spp. beetles (*C. nigropunctata*, *C. undecimnotata*, *C. bistrimaculata*, and *C. bipunctata*). These beetles are known for their highly host-specific natural occurrences (across sites, seasons, and life stages) on their host *Ipomoea* spp. They have strong preferences of hostplants which they can proficiently discern amidst commonly co-occurring close relatives. To find the factors associated with the *Chiridopsis* spp.'s host specificity, we investigated their two key behaviors: 1) hostplant identification and 2) conspecific aggregation on hostplants.

Chapter 2: Determining the cues involved in hostplant location by the *Chiridopsis* spp.

We established the hostplant preferences of the four *Chiridopsis* spp. by studying them in their natural habitats and under laboratory conditions. In all cases, there was a distinct pattern of preference: *C. nigropunctata* was monophagous on *I. elliptica,* and *C. undecimnotata* was biphagous on *I. elliptica* and *I. batatas*. *C. bistrimaculata* and *C. bipunctata* were oligophagous and fed on all *Ipomoea* spp. except *I. parasitica*. We found that plant odor alone was enough to elicit beetle visits to the various *Ipomoea* spp. in the same pattern, suggesting that plant volatiles are the primary hostplant identification cues. To understand the basis of such specialized olfactory resolution, we characterized odor blends of the five *Ipomoea* spp. using GC-MS/-FID and SPME headspace analyses. We found the five odor blends to be composed of a similar set of 29 compounds, but their blends were significantly different based on the concentrations and proportions of these compounds. We identified putative attractants and repellents for each *Chiridopsis* sp. using multivariate statistics, and conducted behavioral assays using these compounds to ascertain their attractant or repellent natures. Beetles responded to these compounds only when they were delivered via their hostplant odor blends; they did not respond when these compounds were given singularly or via non-host odor blends. Using electroantennography, we also ascertained these compounds' perception by the antennal olfactory receptors. From all these experiments, we inferred that these semiochemicals' attractant, repellent, or neutral characters are associated with the hostplant's volatile blend- the matrix. Without the background of all the other co-occurring compounds comprising the hostplant odor, the attractant and repellent compounds do not elicit a behavioral response. We integrated these multi-source data and used a novel tool that we term odor imaging to represent olfactory perceptions as color variations. Odor images revealed beetles' differential olfactory perception of different hostplants and indicated how a beetle could distinguish between two

closely related plant species. Moreover, odor images showed a differential olfactory perception of the same hostplant by different closely-related beetle species. Our results show that the *Chiridopsis* spp, identify hostplants using odor blends rather than individual compounds.

Chapter 3: Deciphering the signals that mediate hostplant-specific aggregation of the *Chiridopsis* spp.

We investigated the conspecific aggregation of the *Chiridopsis* spp. using *C. nigropunctata* and *I. elliptica* as a model system. In nature, we observed that the first visitors on the hostplant initiated the aggregation of conspecifics, and this occurred only after herbivory began. Through behavioral assays, we found that this gregarious behavior occurred even in the absence of visual and tactile cues, suggesting that the aggregation signal was olfactory. Under controlled conditions, aggregation occurred similarly on beetle-devoured and mechanically wounded hostplants and was unaffected by the presence of initiator beetles. These experiments indicated that the signal was not of a beetle origin. We hypothesized that it originated from the beetle-wounded leaves. Temporal analysis of the beetle-devoured and mechanically wounded *I. elliptica* leaf odor blend showed that the wounding induced the emission of three sesquiterpenes, α-copaene, β-copaene, and δ-cadinene, from the leaf. Using complementation assays, we found that α-copaene was the aggregation signal. Beetles responded to α-copaene even without the background hostplant odor. This suggests that like pheromones, they process this signal through the specialized olfactory channels.

Chapter 4: Summary and future perspectives

Our work demonstrated that the *Chiridopsis* species' host specificity is plant-odor-mediated. Hostplant's odor blend matrix is crucial; beetles respond to attractants or repellants only when they are encountered with this matrix. Beetles differentiate closely related host species based on the number and concentrations of attractants and repellants in their blends. Some odorants showed different effects (attracting, deterring or neutral) on different beetle species. This explained why different beetle species had different olfactory perceptions of the same hostplant species. Lastly, we showed that aggregation is also mediated by host-emitted olfactory cue, the wound-induced sesquiterpene. Together, it can be inferred that the closely-related plant species form an ideal system to understand how insects perceive subtle differences between hosts and non-hosts. This multidimensional investigation also underlines the importance of hosts' odor blend fingerprints in the host recognition.

Chapter 1
Introduction

1. Introduction

A large proportion of insects depends on plants for their food, and these plant-insect herbivore interactions are the major conduit for the transfer of energy from plants to higher trophic levels[1]. All insect herbivores exhibit some amount of selectivity in their diet[1]. Often, these evolved diet choices that nutritionally and ecologically support the herbivores, are strictly maintained[2]. Insect diet breadths can greatly vary; those who feed on a wide variety of plant taxa (> one family) are conventionally termed generalists, whereas those who specialize on one hostplant taxon (one family or one species) are termed specialists[3,4]. In all cases, maintaining these specific hostplant associations is crucial for insect survival and requires precise host recognition.

Generally, host recognition is a function of plant chemistry and its interaction with the insect nervous system. Foraging insects begin hostplant identification from a distance while moving through natural habitats. During flight, insects initiate the process using contactless cues that are identifying characters of their hostplant, such as visual or olfactory signals. Plant odor is often a species-specific character[5]. Insects olfactory systems are tuned for detecting and resolving odor cues with high specificity and selectivity[6,7]. Therefore, for insects, hostplant location and identification are mainly enabled with the help of olfaction. After landing on the plant, insects may also make use of tactile and gustatory signals to make the feeding decision. Thus, plant chemistry is instrumental in all the interaction stages. Plant volatiles are of utmost importance for initial identification as they are released into the environment and can be perceived as long-distance cues by the flying insects. Non-volatile metabolites may also be utilized for host selection. However, since they can be perceived only upon contact after landing, they are commonly used as gustatory cues.

Plant odors are the most ubiquitous volatiles in nature[8] and are comprised of diverse volatile organic compounds (VOCs). Commonly found constitutive plant VOCs belong to various classes of compounds, such as isoprenoids (biosynthesized through the mevalonic acid pathway and methylerythritol pathway[9]), fatty acid derivatives including GLVs (biosynthesized through the lipoxygenase/ hydroperoxide lyase pathway[10]), and benzenoids/ phenylpropanoids (biosynthesized from phenylalanine through the shikimate pathway[11]). Plants constitutively produce and release complex VOC blends that give a species its characteristic scent. These constitutive volatile emissions can diffuse through

air and soil[8]. They spatially vary across the plant's life stages and organs and temporally vary across day-time and seasons[12]. The efficient olfactory processing of this information enables insect herbivores to exploit the hostplant at the optimal stage[13].

Plant VOCs are ecologically important. Some VOCs mediate mutualistic interactions as they attract pollinators and seed dispersers, benefitting both the signal emitter and receiver. In many cases, constitutively released VOC cues attract or repel insect herbivores, who utilize these emissions to locate and orient flight or movement towards hostplants for feeding and egg laying. Since plant taxa-specific VOCs are rare, and most plants release ubiquitous volatiles[14], plant odor specificity is likely enabled by the proportions of VOCs in the blend rather than individual compounds. This is supported by studies showing that VOC blends are more attractive to insects than individual components and sometimes blends are attractive while their individual components are not [15–18]. These studies suggest that VOCs can have interactive effects; therefore, an odor blend is an emergent property[7] (discussed further in Chapter 2). The general principles underlying the complex blend effects are unclear[19]. However, there are also examples in the literature where specific VOCs, such as taxa-specific compounds, are associated with hostplant identification. A classic example is isothiocyanates, characteristic of Brassicaceae plants, which insects use as plant recognition cues[12,20]. Apart from hostplant identification, insects also use olfactory cues from their hostplants to aggregate on these food sources. Many insect species display such spatial clustering of conspecifics. It is surmised to reduce interspecific competition and allow resource sharing (discussed further in Chapter 3). The gregarious occurrence can be mediated by attraction to the hostplant via plant VOCs[21,22], or attraction between the conspecifics via hostplant precursor-derived[23,24] or *de novo* synthesized[25] herbivore-emitted aggregation signals.

Plant VOCs are also involved in mediating plant defense and multitrophic interactions. For instance, wounding by herbivores induces a plant response, resulting in an altered VOC emission known as herbivory-induced plant volatiles (HIPVs)[26]. HIPVs are a blend of compounds released after herbivory and may include the compounds that are biosynthesized upon wounding, or upscaled upon wounding[26]. HIPVs include the well-studied green leaf volatiles (GLVs) (six carbon alcohols, aldehydes, and esters), monoterpenes, homoterpenes, and sesquiterpenes. In herbivore-attacked plants, it is known that the release of induced plant VOCs is mediated by the perception of plant cell damage, followed by changes in cytosolic Ca2+ concentration and subsequent signaling

cascade that includes the elicitor methyl jasmonate (MeJA) and phytohormones[26,27]. This signaling can lead to the upregulation of genes in the inducible VOCs' biosynthetic pathways, such as the mevalonic acid pathway that synthesizes sesquiterpenes, and methylerythritol pathway that synthesize monoterpenes and hemiterpenes[9]. Upon herbivory the GLV class of VOCs have also been shown to have increased emissions. This increased emission occurs immediately (1-2 s) after the wounding even in the absence of herbivores, as it is a result of membrane degradation upon mechanical damage to tissues, rather than biosynthetic pathway upregulation[10]. However, upon damage to a plant part, some GLVs such as (Z)-3-hexenyl acetate are also released from intact parts of the attacked plant, suggesting that a systemic signal leads to the activation of the lipoxygenase/ hydroperoxide lyase pathway which synthesizes GLVs[10,28]. The induced volatiles can help defend the plant by deterring insect feeding and oviposition. Additionally, they can attract the herbivore's natural enemies, thus contributing to the plant's indirect defense[29,30]. For instance, the HIPVs released by *Zea mays* L. in response to caterpillar herbivory have been shown to attract the parasitic wasps[31]. In herbivore-attacked lima bean plants, a GLV increases extrafloral nectar synthesis in unattacked leaves of the same plant. The increased nectar attracts ants, which then protect the plant by driving off herbivores[32]. Apart from the wounding while feeding, insect oviposition can also result in altered VOC emissions[12]. On the other hand, when plants face attackers such as microbial pathogens, the VOC response can be different from that induced by herbivores. For example, in peanut (*Arachis hypogaea*), the VOCs released upon infection by the fungal pathogen *Sclerotium rolfsii* include methyl salicylate, 3-octanone and the homoterpene 4,8-dimethylnon-1,3,7-triene (DMNT), whereas herbivory by *Spodoptera exigua* induces only DMNT[33,34]. In natural conditions, plants may face multiple such attackers simultaneously and it may not be metabolically feasible to respond to each stress; the response may then be prioritized based on severity of the attack, or the resources available to cope with each[33]. In addition to these important ecological interactions, plant VOCs can also mediate plant-plant communication. It has been shown that VOCs including volatile phytohormones such as methyl jasmonate, methyl salicylate and ethylene, can be perceived by neighboring plants who are then primed to initiate defense before they are attacked[26,35,36].

How insects perceive plant odor majorly depends on their antennae's repertoire of olfactory receptors. Odorants are perceived upon binding to specialized transmembrane

olfactory receptors (ORs) expressed by olfactory receptor neurons (ORNs) in the sensilla on antennae and mouthparts. However, to reach the transmembrane ORs from the air, the hydrophobic odorants must move through the aqueous sensillum lymph; this is enabled by odorant binding proteins (OBPs)[37]. These ORNs relay information to glomeruli in the antennal lobe; axons of ORNs that express the same OR terminate at the same glomerulus. This information relay allows the chemical information of odor to be converted into a specific glomerular excitation pattern in the antennal lobe[38]. The information in this excitation pattern is then transmitted through a network of local interneurons where the input signal is amplified, and signal to noise ratio is improved. Output neurons then relay the signal to higher brain centers for processing, resulting in behavioral responses such as orientation towards or away from the odor source. Some ORs are narrowly tuned; they are highly specific to an odorant and relay the signal to higher brain centers through dedicated processing channels called labeled lines. On the other hand, some odorants and receptors are broadly tuned or 'promiscuous,' in that one odorant can bind several ORs, and one OR may bind several odorants. Due to this property, different odorant mixtures activate characteristic spatial glomerular excitation patterns, known as combinatorial coding. This type of olfactory processing may allow insects to discriminate between a large number of plants and increase the perceived odor space using a limited OR repertoire.

Studies on hostplant identification majorly involve single model or crop species, often agricultural pests foraging in areas of hostplant monocultures. However, natural ecosystems contain mixed vegetation, where closely-related plants with similar chemical repertoires frequently co-occur. This creates a complex odorscape with high background noise which a forager must navigate to precisely locate their hosts. How insects resolve such complex cue mixtures is not clearly understood. To understand how this occurs in such habitats with complex cue mixtures, we must explore natural systems. In this project, we studied the olfaction-mediated hostplant specialization in a native plant-insect herbivore system from the Western Ghats: five plants of the genus *Ipomoea* (*I. batatas*[39], *I. carnea*[40], *I. elliptica*[41–44], *I. triloba*[45], and *I. parasitica*[46]) and their four specialist *Chiridopsis* spp. beetles (*C. nigropunctata*[47], *C. undecimnotata*[48], *C. bistrimaculata*[47,48], and *C. bipunctata*[48]). Previous observations in the forests were that the insects' natural occurrence on the *Ipomoea* spp. followed a consistent pattern, suggesting a high level of host specificity. We studied the plant-associated factors associated with two key

behaviors in the insects: host identification (Chapter 2) and aggregation at the food source (Chapter 3).

Chapter 2

Determining the cues involved in hostplant location by *Chiridopsis* spp.

2. Determining the cues involved in hostplant location by the *Chiridopsis* spp.

2.1 Introduction

Plant-herbivore food webs represent more than 40% of global terrestrial biodiversity, and a large majority of these herbivores are phytophagous insects[49]. The range of hostplants occupied by insect herbivores shows large variability, from one or few plant taxa to several different plant taxa. Hostplant selection by phytophagous insects holds significant importance in an ecological system because this variation among organisms influences several ecological phenomena, such as the coexistence of competitors[50], persistence of species upon environmental disturbances[51], and maintenance of inter-species interaction networks[52,53]. Locating and identifying hostplants amid complex mixed vegetation is a challenging but crucial task for insects[4] and is facilitated by a combination of sensory inputs. Recognizing a plant commences with perceiving olfactory and visual cues from a distance, followed by gustatory and tactile cues that may help host selection after contact[1]. Insects incorporate these various sensory inputs into forming a foraging decision; upon perceiving the correct combination of indicators, a plant is recognized as palatable or unpalatable[4,54].

Of all these sensory signals, plant volatile organic compounds (VOCs) play a significant role in mediating this interaction as they are often used by insects to identify hosts and non-hosts while in flight[2,55–58]. In fact, VOCs are known to be used for host identification even by insect herbivore larvae[59–62], ovipositing females[63–67], parasitoids[57,68–70], and even in underground interactions[71]. Research has explored the effects of individual plant VOCs or groups of VOCs on insect behavior. Several studies have successfully identified species-specific plant VOCs that are attractants or repellents for their insect herbivores[61,72–75,75–78]. On the other hand, other reports suggest that it is the blend of all the released compounds which insects collectively perceive while making a host selection/ location decision[14,16,79,80]. To understand hostplant location and identification amidst mixed vegetation, studying plant odor blends rather than single compounds offers a more realistic portrayal of what foraging insects encounter. Plant odor blends are often complex mixtures consisting of hundreds of compounds[56,58,81]. Most identified plant volatiles are ubiquitous across plants as odorants characteristic of plant taxa are rare[58,82–84], and there is a broad overlap between the odorants that different insects can detect[58,85].

This odor detection overlap within a limited range of plant VOCs, suggests that the identification is enabled the combination of compounds and their signal processing in the insect nervous system. Therefore, it is natural for a foraging insect that the functional unit of plant odor is not a single compound but multiple compounds co-occurring in a blend. There is also evidence that subtle alterations in the proportions of these compounds can drastically affect the host location behavior[7,86,87]. Further, specific mixtures of volatiles have been demonstrated to attract insects when their individual components did not[17], and compounds functioning as host cues in a blend have been shown to become non-host cues when presented alone[15]. All these reports support the idea that odor blends have emergent properties, and that hostplant identification relies on recognizing the blend rather than individual components[7,15,88]. However, not all volatile components of an odor blend may be relevant to foraging insects; only compounds that can be perceived by the insects' olfactory receptors can lead to neuron firing and eventually be integrated into the central nervous system to bring about a behavioral response[6]. Whether a volatile compound can be perceived by insect antennae is usually tested by the electrophysiological response analyses such as electroantennography (EAG) that records electric potentials across the antenna, or single sensillum recordings (SSR) which records electrical activity elicited by ORNs in a single sensillum[89]. In EAG, an excised insect antenna is placed between two electrodes connected to an amplifier. The antenna is exposed to an air pulse carrying the test volatile compound. If the insect's antenna bears olfactory receptors for the test compound, an electrical signal, a microcurrent, is generated. It is then amplified and recorded as an antennal response to the test compound[90].

Researchers have studied how an insect simultaneously perceives all the components of odor blend and which features are critical for host recognition. These chemical cues have mainly been studied using a single agricultural pest model or crop species, where large areas of hostplant monocultures are grown[5]. In comparison, insects' original ecosystems are much more diverse, where they encounter complex odor bouquets from different plant communities, including abundant non-host odor and high background noise[7,15,56,91]. Moreover, such mixed vegetation habitats frequently harbor closely-related co-occurring plant species with similar chemical repertoires. To understand how foraging insects resolve these complex cue mixtures, we must study wild plant-insect systems in natural habitats. In this regard, a wild system of closely related plants and insects can offer much

valuable insight into understanding host recognition in natural odorscapes. The tortoise beetles and their larvae (Coleoptera: Chrysomelidae), specialist folivores of the plant family Convolvulaceae, offer such a system. Our observations in the northern ranges of the Western Ghats were that among all the genera of these cassidine beetles, species of genus *Chiridopsis* show exclusive hostplant associations with plants of the genus *Ipomoea*. For example, *C. nigropunctata* is monophagous (feeds on only one *Ipomoea* species), *C. undecimnotata* is biphagous (feeds on two *Ipomoea* species), whereas *C. bistrimaculata* and *C. bipunctata* are oligophagous (feed on more than two *Ipomoea* species). A naturally available negative control in the study system is *I. parasitica*, which co-occurs with the other plant species, but is not fed upon by these beetles. The *Ipomoea-Chiridopsis* system is a unique interaction in which such the hostplant spectrum of one insect genus is exclusively associated with different species of only one hostplant genus. Even when these congeneric plant and insect species co-occur, we have observed that when beetles are disturbed, they start flying around and return to their hostplant often without landing on the neighboring plants of other species. The *Ipomoea-Chiridopsis* system can thus be ideal to understand cues involved in host identification and selection. Specifically, we asked, upon encountering these closely related plants during foraging, how do these beetles perceive and differentiate the odors of the closely related host and non-host *Ipomoea* plants?

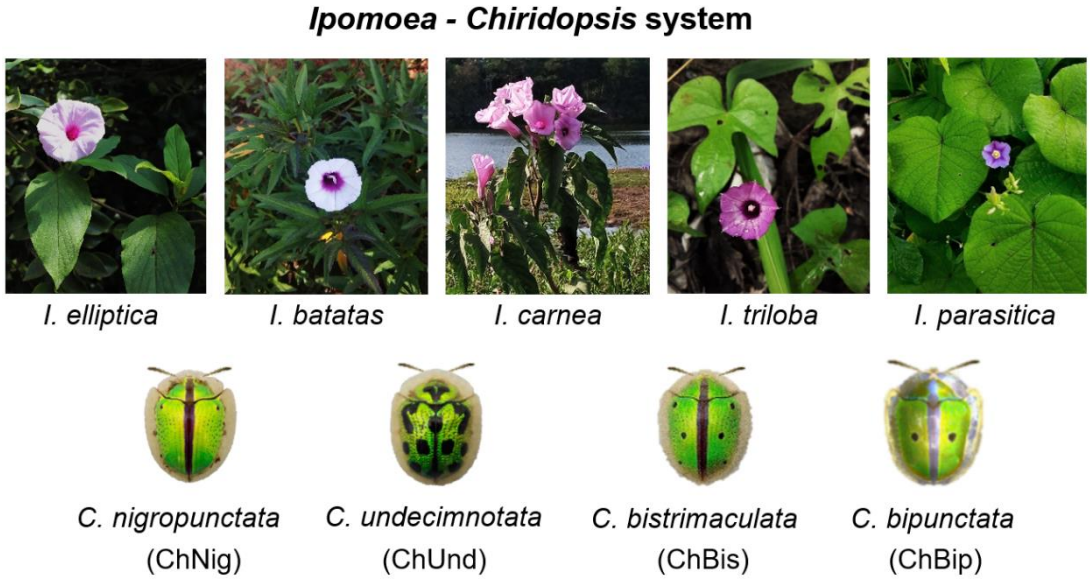

Figure 2.1: Groundwork: The *Ipomoea-Chiridopsis* system.

2.2 Materials and methods

2.2.1 Plants

Seeds of *I. batatas, I. carnea, I. elliptica, I. triloba,* and *I. parasitica* were collected from in and around Pune. Plants were grown and maintained in controlled conditions (temperature: 27°C, humidity: 70%, photoperiod: 12 h light and 12 h dark) in a climate chamber. Fresh leaves from grown plants were used for insect behavioral assays, VOC profiling, and maintaining insect cultures in the laboratory. In behavioral assays, fully expanded healthy, unwounded leaves were used, unless otherwise specified.

2.2.2 Insects

Adults and larvae of *C. nigropunctata, C. undecimnotata, C. bistrimaculata,* and *C. bipunctata* were collected from in and around Pune. Insects were reared on fresh leafy twigs of their host plants in an insectarium with the same controlled conditions as the climate chamber.

2.2.3 Field observations on natural occurrence

To understand the hostplant preferences of *Chiridopsis* insects, we observed their occurrence on the five *Ipomoea* spp. in their natural habitats of the Western Ghats. Observations were made in three separate sites in three seasons (n= 9). In every site, the total number of ootheca, larvae, and beetles of each species were counted on 10 plants of each *Ipomoea* sp.

2.2.4 Multiple-choice assays

To understand the feeding preference of *Chiridopsis* beetles in multiple-choice assays, we presented each *Chiridopsis* spp. with fresh leaves of all five *Ipomoea* spp. in an assay jar (height 15 cm, diameter 25 cm). An artificial leaf of average surface area (adaxial+ abaxial) 50± 5 cm^2 weighing 0.3± 0.05 g (similar to the average surface area and weight of an *I. elliptica* leaf) was cut from Whatman filter paper and included among the choices as a negative control. For each *Chiridopsis* sp., we conducted six assays (n= 6). Considering these insects' slow feeding rates, we standardized the assay time to be 24 h to provide beetles enough time to explore and feed on all given choices with a quantifiable area. We calculated the amount of feeding on each leaf as [(leaf area devoured from a given *Ipomoea* spp./ total area devoured from all leaves) × 100].

2.2.5 No-choice assays

To understand the feeding behavior in the absence of the most preferred hostplant, we conducted no-choice assays for each beetle species, where one individual was exposed to a fresh leaf of a single *Ipomoea* sp. at a time. Each assay consisted of 5 caged with a single *Ipomoea* sp. for 6 h (n= 30). We calculated the amount of feeding on each *Ipomoea* sp. as surface area devoured (mean± SE).

2.2.6 Survivorship assays

To study the survivorship of insects on the different hosts, we released 20 individuals of each *Chiridopsis* sp. (per plant) on each *Ipomoea* sp. (n= 5). Plants were caged to prevent insects from escaping. Insects were allowed to feed on the plants for five days. We counted the survivor number on each plant in each assay and calculated the survivorship as [(number of survivors on a given *Ipomoea* sp./ 20) × 100] (mean± SE).

2.2.7 Total odor blend complementation assay

Volatile organic compounds were extracted from the fully expanded, healthy unwounded leaves of *I. batatas*, *I. carnea*, *I. elliptica*, *I. triloba*, and *I. parasitica* by solvent extraction method. In a screw-cap glass vial with silicone septa, 5 mL dichloromethane (DCM) was used to extract volatiles from 1 g of leaf tissue for 2 h. Extracts were dehydrated using anhydrous sodium sulfate (Rankem, India) and further concentrated to 1 mL using a vacuum concentrator (Labconco, Kansas City, MO, USA). Concentrated extracts were incubated overnight at -80°C to precipitate high molecular weight lipids, which were then removed by centrifugation at 10000 rpm for 10 min at 4°C. Extracts were further concentrated to a final volume of 250 µL and stored in air-tight glass autosampler vials (Chromatography Research Supplies, India) at -20°C till further use. These DCM extracts majorly contain plant odorants; therefore, henceforth the *Ipomoea* leaf extracts will be referred to as 'odor blends.' We complemented five artificial leaves each with the odor blend of a single *Ipomoea* sp. (physiological concentration). Every *Chiridopsis* spp. was subjected to multiple-choice assays between the five odor-complemented leaves (n= 20). A DCM-complemented and a non-complemented artificial leaf were included in each assay as controls. Beetle visits on each artificial leaf were quantified to understand whether plant odor is the primary hostplant identification cue.

2.2.8 Extraction and analysis of *Ipomoea* odor blends

We extracted VOCs from *I. batatas, I. carnea, I. elliptica, I. triloba,* and *I. parasitica* leaves by the solvent extraction method described earlier. As an internal standard for quantification, we spiked the extraction solvent DCM with nonyl acetate (2.2 µg/ mL). Compounds were identified and quantified using a gas chromatograph (7890B GC system, Agilent Technologies) coupled to a mass spectrometer and flame ionization detector (7000D GC/triple quadrupole and FID, Agilent Technologies, Santa Clara, CA, USA). Compounds were separated on a DB-5MS capillary column (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Agilent Technologies) using helium as carrier gas with a flow rate of 2 mL/ min. Column temperatures were programmed as follows: 40 °C hold time 5 min, ramp 1: 5 °C/ min till 180 °C, ramp 2: 20 °C/ min till 280 °C, hold time 5 min. Mass spectra were obtained using 70 eV electron ionization with a scan time of 0.2 s for m/z 30- 600. Compounds were identified using mass spectral libraries NIST11 and Wiley (8th edition). Kovat's retention indices for compounds were calculated using an n-alkane ladder (C7-C21). Compounds were quantified on GC-FID, where concentrations of different volatiles were normalized with nonyl acetate[92].

2.2.9 Statistical analyses

Quantitative data (number of insects on different *Ipomoea* spp., feeding preference, and survivorship on different *Ipomoea* spp.) were analyzed by one-way analysis of variance (ANOVA), and the statistical significance ($p \leq 0.05$) was determined by Fisher's least significant difference on StatView software (ver. 5.0).

We performed a principal component analysis (PCA) to understand how the five *Ipomoea* spp. differ in their VOCs. PCA was performed on a correlation matrix to account for scale differences in various compounds. To understand whether the *Ipomoea* spp. form significantly different clusters with respect to their VOCs, we performed an analysis of similarity (ANOSIM) using Manhattan distances to account for high dimensionality in the data. ANOSIM was performed with 9999 permutations at two levels, at the level of all groups together with the null hypothesis that all groups are the same and pairwise comparison between groups. When multiple pairwise tests were performed, we used sequential Bonferroni correction to account for family-wise errors. Analysis was performed using PAST 3.26.

We performed a partial least square (PLS) analysis to understand the correlation between multiple dependent variables (feeding preferences of *Chiridopsis* spp.) and multiple independent variables (plant volatiles). The null hypothesis was that there is no correction between the given variables and the first and second axes of PLS, and this was tested using one-sample t-test. Multiple regression between the feeding preferences of *Chiridopsis* spp. and *Ipomoea* volatile compounds was performed to understand whether the plant VOCs determine the observed feeding preferences. The null hypothesis that the standardized coefficient of the multiple regression was not significantly different from zero was tested using multiple one sample t-tests. We used sequential Bonferroni correction to account for family-wise errors. Analysis was performed using XLSTAT®.

2.2.10 Complementation assays

To understand the function of compounds correlated with *Chiridopsis* hostplant identification, we conducted complementation assays with all candidate compounds commercially available as analytical standards.

Complementation of individual compounds on artificial leaves

For each *Chiridopsis* spp., each putative attractant and repellent was complemented on an artificial leaf in serially increasing concentrations (n= 20 for each concentration). The first concentration pasted was equal to half the physiological concentration in the most-preferred host. The next used concentration was equal to the physiological concentration, thereby doubling the compound's concentration in the test leaf (2-fold increase). Further increments were 4, 6, 8, and 10 folds. In addition to assays using the compounds correlated with beetle preference, we also performed assays using the following technical controls:

- Neutral compound: compound detected in *Ipomoea* spp. but not correlated to beetles' preferences (β-caryophyllene)
- Foreign compounds: Compounds not detected in any of the five *Ipomoea* spp. Three foreign compounds were used: hexanal (a green leaf volatile), *(Z)*-3-nonen-1-ol (an aliphatic alcohol), and valencene (a sesquiterpene).

At each increment, we recorded beetles' preferences using a dual choice assay (1 h duration) including a compound-complemented (test) and solvent-complemented (control) leaf choices. To estimate attraction towards or deterrence from the test leaves,

we calculated the percentage of beetles preferring test and control leaves on their first visits (mean± SE).

Complementation of individual compounds on host and non-host leaves

To find how these compounds operate within a blend, we serially raised the concentration of each attractant and repellent in two most-preferred plants and two least-preferred plants of each *Chiridopsis* sp. (n= 10 per concentration). This was done by exogenously applying the compound over the leaf in increasing concentrations, thereby raising its total concentration in the test leaf to 1.5, 2, 4, 6, 8, and 10 folds. At each increment, we observed beetles' preference for 1 h in a dual choice assay between a test and control leaf. Preference for a choice was calculated as the percentage of beetles who visited and initiated feeding on it (mean± SE).

Complementation of individual compounds on artificial leaves along with host and non-host odor blends

We also applied attractants and repellents in increasing concentrations as described earlier (n= 10 per concentration) on artificial leaves along with odor blends of the most-preferred plants or the non-host *I. parasitica.* In these assays, the filter paper artificial leaves used were cut in the shape and size of *Ipomoea* leaf in consideration. At each increment, we observed beetles' preference for 1 h in a dual choice assay between a test and control leaf. Preference for a choice was calculated as the percentage of beetles who visited and initiated feeding on it (mean± SE).

2.2.11 Solid phase microextraction (SPME) headspace analysis

Presence of the candidate compounds in the *Ipomoea* headspace was ascertained by SPME. For each species, a potted plant was enclosed in a ventilated glass cylinder, and exposed to an SPME fiber assembly (divinylbenzene/ carboxen/ polydimethylsiloxane, needle size 24 ga; Sigma, India) for 1 h to collect headspace volatiles. Soil was covered with a polypropylene bag to minimize release of soil volatiles into the headspace. The same setup without a plant was used as a blank.

2.2.12 Electroantennography

Electrophysiological response of beetle antennae to the candidate compounds was tested using an electrographic system (Syntech, Hilversum, The Netherlands) consisting of a dual electrode probe for antenna fixation, a CS-05 stimulus controller and an IDAC 232

box for data acquisition. Each antenna was fixed between the two electrodes using Spectra 360 conductive gel (Parker, Orange, New Jersey) as suggested by Reinecke *et al.*[90]. The antenna was continuously flushed with a stream of activated charcoal-filtered air. Solutions of authentic standards were prepared in DCM and 10 µL from each was applied to a filter paper strip. The solvent was allowed to evaporate before placing the strip in the apparatus. A purified airstream (pulse time 0.5 s, continuous flow 25 ml/ s, pulse flow 21 ml/ s) flowing over the antennal preparation delivered the stimulus puff. A time delay of 20 s was maintained between consecutive stimulus puffs. The antennal responses were recorded through a high impedance probe connected to amplifier (IDAC-4, Syntech), as voltage deflections (mV). The blank stimulus was DCM. 10 replicates each were done for six concentrations (0.625, 1.25, 2.5, 5, 10, and 20 ppm) of all compounds. Each EAG response was corrected for solvent and background effects by subtracting the response to DCM from the response to the stimulus.

2.2.13 Odor imaging

To visualize olfactory perceptions as color variations, we generated odor images for every beetle-plant pair. For this, we multiplied the concentration of each attractant, repellent, and neutral compound with the beetle's standardized regression coefficient for that compound. The resulting values were plotted as a square pie diagram for every beetle-plant pair. Leaf shapes were pixelated using the pie charts such that each pixel was a pie chart.

2.3 Results

2.3.1 Occurrence of *Chiridopsis* spp. on *Ipomoea* spp. shows a distinct preference pattern in nature

Our field observations were that the four beetle species occur only on certain *Ipomoea* spp. (Fig. 2.2A), exhibiting a distinct pattern. *C. nigropunctata* was found only on *I. elliptica* (Fig. 2.2B-D), and *C. undecimnotata* was found only on *I. elliptica* (majorly) and *I. batatas* (Fig. 2.2E-G). On the other hand, *we found C. bistrimaculata* and *C. bipunctata* on four hostplants- *I. batatas, I. carnea, I. elliptica,* and *I. triloba*, displaying preference for some over others (Fig. 2.2H-M). We did not observe any ootheca, larvae, or beetles on *I. parasitica*. The hostplant-specific occurrence of each *Chiridopsis* sp.

showed the same trend at various developmental stages such as ootheca, larvae, and adults.

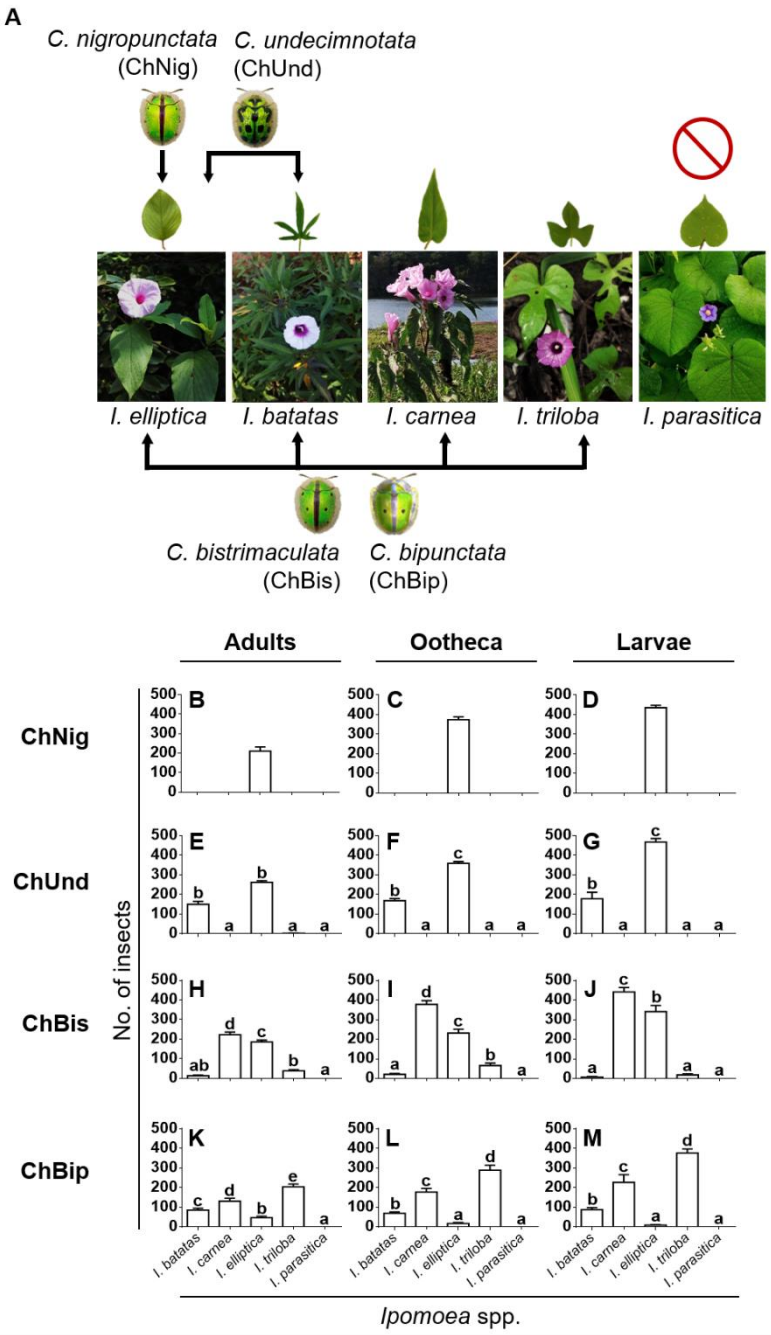

Figure 2.2: Natural occurrence of *Chiridopsis* spp. on *Ipomoea* spp. (**A**) Arrows indicate occurrence. Beetle names have been given abbreviations which will be used throughout the figures. Observations from the wild are that there is a high specificity in the co-occurrence of each *Chiridopsis* spp. with specific *Ipomoea* spp. No *Chiridopsis* spp. has been found on *I. parasitica*. Occurrence of adults, ootheca and larvae of (**B**) to (**D**) *C. nigropunctata*, (**E**) to (**G**) *C. undecimnotata*, (**H**) to (**J**) *C. bistrimaculata* and (**K**) to (**M**) *C. bipunctata* was measured on different *Ipomoea* plants in their natural habitats. Insect numbers were counted in 3 field locations in 3 seasons (n= 9). In each location, the number of insects on each *Ipomoea* sp. was considered as the total on ten individuals. Data is plotted as mean± SE. Hostplant preferences of each species followed the same trend in every stage of its life cycle. Different letters denote significant difference ($p \leq 0.05$, one-way ANOVA).

2.3.2 *Chiridopsis* spp. maintain stringent hostplant preferences under controlled conditions

We conducted choice and no-choice assays under controlled laboratory conditions to experimentally validate our field observations. When presented multiple choices simultaneously, feeding preferences of the *Chiridopsis* spp. (percentage area fed on each leaf in the assay) closely followed the trend of their natural occurrences (Fig. 2.3A-D). *C. nigropunctata* was strictly monophagous on *I. elliptica* (Fig. 2.3A), whereas *C. undecimnotata* fed only on *I. elliptica* and *I. batatas*, preferring the former nearly five times more than the latter (Fig. 2.3B). *C. bistrimaculata* and *C. bipunctata* were oligophagous on *I. elliptica, I. batatas, I. carnea,* and *I. triloba* (Fig. 2.3C, D). However, they exhibited some preferences: *I. carnea* was most preferred and *I. batatas* was least preferred by *C. bistrimaculata*. Contrarily, *C. bipunctata* most preferred *I. batatas* and least preferred *I. carnea.* In all assays, no beetle fed on *I. parasitica*. We also conducted no-choice assays to observe whether these patterns are altered upon the unavailability of the preferred plants. In almost all cases, *Chiridopsis* beetles maintained their hostplant range and showed similar preferences as described above (Fig. 2.3E-H). *C. nigropunctata* remained monophagous on *I. elliptica* and strictly avoided all other plants. When provided its two hostplants separately, *C. undecimnotata* equally fed on both, not displaying a preference for *I. elliptica* over *I. batatas*. Although scantily, it also fed on *I. triloba* (a non-host in nature). The oligophagous *C. bistrimaculata* fed on all hostplants without a preference for one. *C. bipunctata*, however, maintained *I. batatas* and *I. carnea* as its most preferred and least preferred host respectively. *I. parasitica* remained as a non-host.

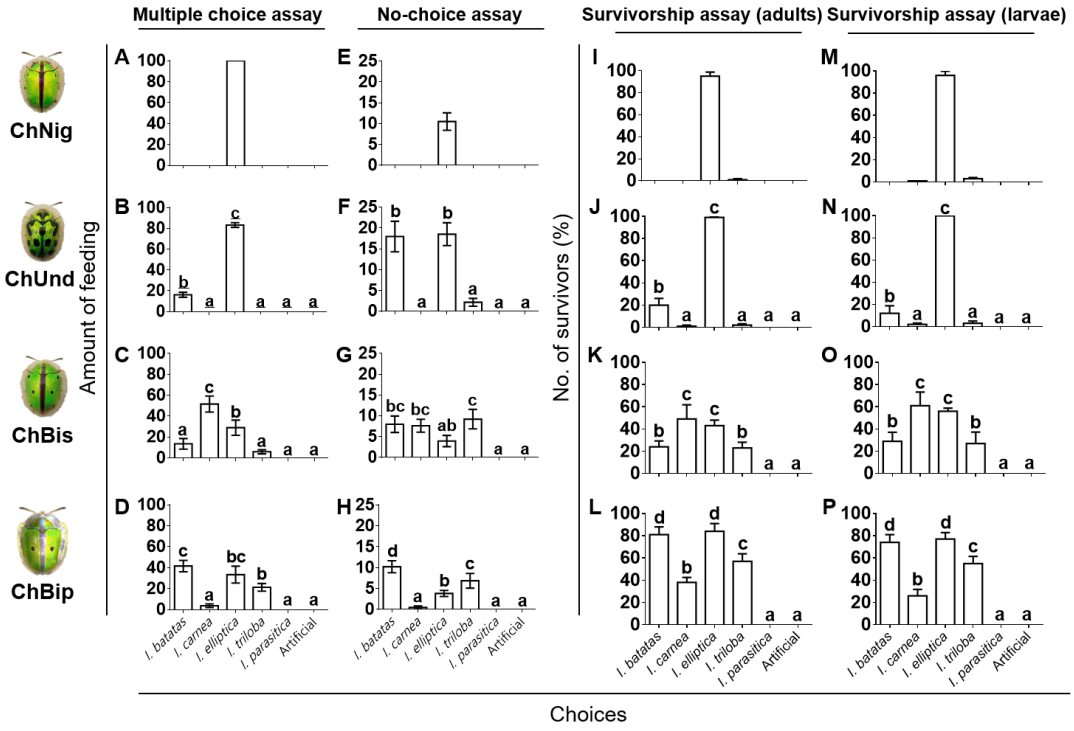

Figure 2.3: *Chiridopsis* spp. show highly stringent hostplant preferences in feeding. (**A**) to (**D**) Multiple choice assays established the mono-, bi-, and oligophagous nature of the *Chiridopsis* spp. Hostplant preferences were measured as percentage area (mm²) fed on each *Ipomoea* sp. leaf in 24 h (mean± SE, n= 6). (**E**) to (**H**) No-choice assays confirmed that the hostplant associations of the *Chiridopsis* spp. do not change even in absence of most preferred hostplants. Amount of feeding was measured as area (mm²) fed on each *Ipomoea* sp. leaf (mean± SE, n= 30). Feeding preferences of each *Chiridopsis* spp. shows the same trend as their survivorship on the different plants, in both adult (**I**) to (**L**) and larval (**M**) to (**P**) life stages (n= 5 assays, each containing 20 insects). Different letters in graphs denote significant difference ($p \leq 0.05$, one-way ANOVA).

2.3.3 Insects' hostplant preferences follow the trend of their survivorship

In survivorship assays, we observed the percentage of larval and beetle survivors to be highest on their respective most-preferred hosts and lowest on least-preferred hosts (Fig. 2.3I-P). *C. nigropunctata* and *C. undecimnotata* had 100% survivorship on their most preferred hostplant, *I. elliptica* (Fig. 2.3I, J, M, N), on which they fed heavily. On non-hosts, most *C. nigropunctata* and *C. undecimnotata* insects refrained from feeding and died of starvation. Concurrent with above trends, *C. bistrimaculata* and *C. bipunctata* fed and survived on all *Ipomoea* spp. except *I. parasitica* (Fig. 2.3K, L, O, P). For these oligophagous insects, 100% survivorship was not observed on any hostplant. Despite this, the survivorship on different plants closely resembled the trend of their feeding choice. Survivorship of *C. bistrimaculata* was the highest on its most preferred host *I. carnea,* and lowest on its least preferred host *I. batatas*. Survivorship of *C. bipunctata* was highest

on its most preferred host *I. batatas*, and lowest on its least preferred host *I. carnea*. We also observed 100% mortality in all insects exposed to *I. parasitica*, with no trace of feeding on the plants. The low survivorship observed in all the *Chiridopsis* spp. on their least preferred hostplants suggests that although these plants are palatable, the ingested leaf material may contain unfavorable or toxic components that negatively affect the insect's survival on that plant. This suggests the possible role of non-volatile hostplant signals, such as gustatory cues[93], that the *Chiridopsis* spp. may be using to make feeding decisions after landing on such hosts.

2.3.4 Hostplant odor is the major host identification cue

When each *Chiridopsis* sp. was simultaneously subjected to the odor extracts of the five *Ipomoea* spp. (Fig. 2.4A), beetles paid more visits to artificial leaves complemented with their hostplants' odor than non-complemented ones (Fig. 2.4B-E). The trend closely followed the trend of feeding preferences, with visits being paid to the most preferred and to the least preferred hostplant odor. No visits were paid by any beetle to non-host odors. The observation that hostplant odor alone was sufficient to prompt beetle visits suggest that host identification for the *Chiridopsis* spp. is strongly associated with olfactory cues.

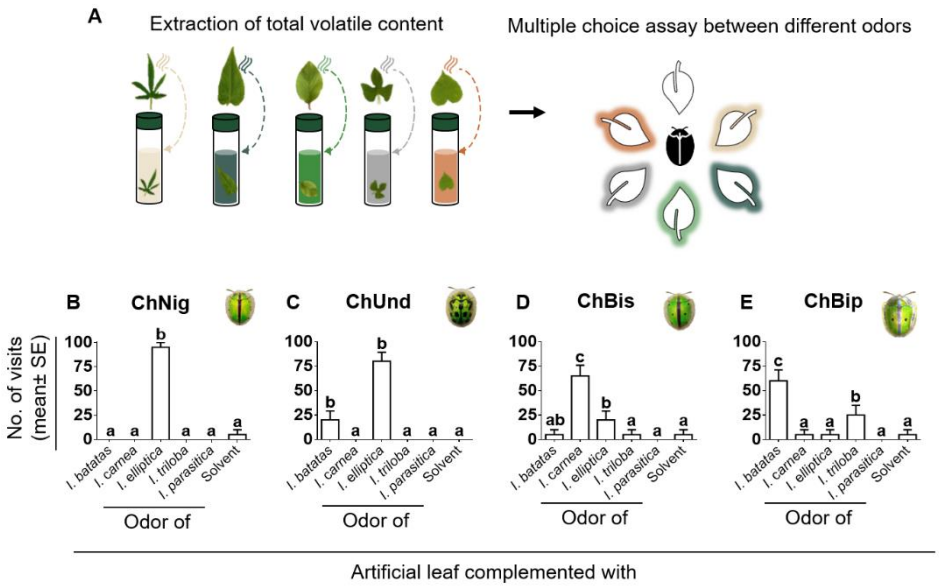

Figure 2.4: Olfactory signals are the major cues used by *Chiridopsis* spp. for hostplant identification. (**A**) Schematic of odor blend complementation assay. Total volatile blend was extracted from each *Ipomoea* spp. and pasted on artificial leaves. Each beetle's preference was assayed when simultaneously provided artificial leaves complemented with different odor blends. An artificial leaf coated with solvent was used as control. (**B**) to (**E**) Number of visits to each plant's blend closely resembles the trend of feeding preferences. Different letters denote significant difference ($p \leq 0.05$ respectively, one-way ANOVA, n= 20).

2.3.5 *Ipomoea* spp. produce characteristic odor blends

Leaf volatile organic compounds (VOCs) of the five *Ipomoea* spp. were extracted and analyzed by gas chromatography-mass spectrometry and gas chromatography-flame ionization detection (GC-MS and GC-FID). We identified 29 compounds, each present in unique combinations and concentrations in each *Ipomoea* sp., thereby generating five signature odors (Table 1, Table 2, Fig. 2.5A). Compounds were identified based on their Kovat's indices (Table 2). For all species, sesquiterpenes contributed to majority of their odor (90.8- 95.9%). The quantitative contribution of aldehydes (0.2- 3.8%), oxygenated terpenes (0.02- 1%), and other compounds (0.5- 1.9%) to the blends was relatively minor. Total volatile content was highest in the herbivore-resistant *I. parasitica* (3655.13 ± 194.37 nmol/ g leaf). The sesquiterpene germacrene-D was the most quantitatively dominant compound in all five odor blends. *I. parasitica* contained ≥ 3.9-fold higher concentration (1433.6± 145.45 nmol/ g) than the other *Ipomoea* spp. (Table 1). We performed a principal component analysis (PCA) and analysis of similarity (ANOSIM) using Manhattan distances to understand how these five odor compositions compare with each other. PCA separated the five *Ipomoea* species based on their VOCs on the first two axes (Fig. 2.5B). On the first PCA axis, *I. batatas* and *I. parasitica* separated from *I. carnea, I. elliptica,* and *I. triloba* based on high factor loading for volatile compounds like *(Z)*-hex-3-en-1-ol, γ-elemene, β-cubebene, β-elemene, β-caryophyllene, β-copaene, germacrene-D, and bicyclogermacrene, and low factor loading for compounds like camphol and 1,3-ditertiary-butylbenzene. Clusters of plant species with respect to their VOC composition were significantly different (ANOSIM, R= 0.7565, *p*= 0.0001) (Table 3).

Table 1 | Volatile compounds detected in *Ipomoea* spp. Concentration of 29 volatile compounds identified from *I. batatas, I. carnea, I. elliptica, I. triloba* and *I. parasitica* (nmol/ g leaf tissue, mean± SE, n= 6). Reported concentrations are estimated by normalizing relative to nonyl acetate internal standard. nd= Not detected in analysis.

Compound			Concentration in *Ipomoea* spp. (nmol/ g, mean± SE)				
Class	**Name**	**Abbreviation**	***I. batatas***	***I. carnea***	***I. elliptica***	***I. triloba***	***I. parasitica***
Alcohols	Benzyl alcohol	BOL	4.51 ± 0.42	2.87 ± 0.48	nd	nd	2.51 ± 0.26
	Phenylethyl Alcohol	PEA	44.81 ± 5.76	3.96 ± 0.36	nd	nd	nd
	β-Phenoxyethyl alcohol	βPEA	2.6 ± 0.19	nd	nd	nd	nd
	(E)-Hex-3-en-1-ol	EHOL	8.29 ± 0.3	nd	nd	nd	nd
	(Z)-Hex-3-en-1-ol	ZHOL	64.36 ± 5.33	35.91 ± 7.65	30.63 ± 4.33	70.06 ± 9.93	111.25 ± 15.57
Aldehydes	Benzeneacetaldehyde	BAL	8.29 ± 0.93	11.97 ± 2.59	19.26 ± 2.24	8.52 ± 1.04	9.02 ± 1.04
	3-Hexenal	HAL	15.61 ± 0.35	nd	25.66 ± 3.02	nd	nd
Oxygenated terpenes	Camphol	CAM	0.33 ± 0.04	0.43 ± 0.05	0.36 ± 0.03	0.39 ± 0.02	0.28 ± 0.02
	Germacrene D-4-ol	GOL	3.86 ± 0.42	4.13 ± 0.83	11.78 ± 3.25	nd	11.42 ± 1.37
Sesquiterpenes	γ-Elemene	γELE	136.25 ± 19.8	nd	15.34 ± 3.56	258.81 ± 22.92	260.19 ± 25.89
	δ-Elemene	δELE	nd	83.11 ± 14.47	nd	nd	nd
	α-Copaene	αCOP	23.29 ± 2.49	2.08 ± 0.39	45.89 ± 12.39	42.87 ± 3.8	11.17 ± 1.42
	β-Cubebene	βCUB	9.66 ± 1.56	1.5 ± 0.1	1.65 ± 0.38	4.65 ± 0.31	7.07 ± 1.32
	β-Elemene	βELE	115.24 ± 12.84	16.85 ± 1.54	19.91 ± 4.95	27.28 ± 2.07	126.02 ± 14.72
	β-Caryophyllene	βCAR	542.51 ± 57.21	206.11 ± 24.91	123.54 ± 22.02	194.67 ± 20.45	912.03 ± 112.38
	β-Copaene	βCOP	29.47 ± 3.03	24.97 ± 5	21.3 ± 8.13	20.15 ± 2.24	77.92 ± 8.07
	α-Bergamotene	αBER	68.48 ± 5.29	17.94 ± 3.95	36.01 ± 13.06	2.69 ± 0.38	8.11 ± 0.84
	Isogermacrene D	IGD	5.7 ± 0.4	12.61 ± 2.45	12.23 ± 4.4	8.94 ± 0.91	32.54 ± 3.3
	Humulene	HUM	80.5 ± 7.95	38.03 ± 4.4	129.67 ± 22.63	37.83 ± 3.92	182.4 ± 21.14
	epi-β-Caryophyllene	eβC	nd	nd	134.71 ± 55.44	nd	nd
	Germacrene D	GD	543.49 ± 52.23	463.2 ± 93.22	388.06 ± 148.05	367.28 ± 42.24	1433.55 ± 145.45
	Bicyclogermacrene	BCG	175.6 ± 24.68	21.29 ± 4.17	80.66 ± 29.71	409.49 ± 35.11	439.63 ± 48.17
	α-Farnesene	αFAR	14.24 ± 0.78	32.44 ± 7.42	nd	27.14 ± 2.66	11.68 ± 3.26
	β-Cadinene	βCAD	nd	nd	15.68 ± 5.88	nd	nd
	δ-Cadinene	δCAD	13.69 ± 0.94	2.46 ± 0.37	49.37 ± 29.69	18.25 ± 1.27	1.96 ± 0.36
	Germacrene B	GB	3.48 ± 0.28	3.67 ± 0.45	nd	nd	nd
Other	1,3-Di-tertiary-butylbenzene	DTB	13.27 ± 2.34	13.29 ± 1.1	19.46 ± 0.77	21.6 ± 1.46	11.78 ± 1.01
	(Z)-3-Hexenyl Acetate	ZHAC	1.44 ± 0.26	5.55 ± 1.28	2.35 ± 0.44	nd	4.61 ± 0.66
	Eugenol	EUG	4.81 ± 0.52	1.14 ± 0.1	nd	nd	nd
Total concentration			1933.8 ± 85.71	1005.51 ± 98.65	1183.51 ± 168.17	1520.62 ± 64.12	3655.13 ± 194.37

Table 2 | Compounds with their experimentally determined and reported Kovat's retention indices (KI).

Name	Compound	KI experimentally determined	KI reported on NIST MS library
Benzyl alcohol	BOL	1043	1036
Phenylethyl Alcohol	PEA	1119	1116
β-Phenoxyethyl alcohol	βPEA	1228	1225
(E)-Hex-3-en-1-ol	EHOL	866	852
(Z)-Hex-3-en-1-ol	ZHOL	861	861
Benzeneacetaldehyde	BAL	1050	1045
3-Hexenal	HAL	803	810
Camphol	CAM	1172	1167
Germacrene D-4-ol	GOL	1494	1574
γ-Elemene	γELE	1434	1434
δ-Elemene	δELE	1344	1338
α-Copaene	αCOP	1382	1376
β-Cubebene	βCUB	1396	1389
β-Elemene	βELE	1397	1391
β-Caryophyllene	βCAR	1416	1419
β-Copaene	βCOP	1451	1432
α-Bergamotene	αBER	1423	1415
Isogermacrene D	IGD	1427	1448
Humulene	HUM	1434	1454
epi-β-Caryophyllene	eβC	1439	1440
Germacrene D	GD	1451	1481
Bicyclogermacrene	BCG	1457	1495
α-Farnesene	αFAR	1460	1508
β-Cadinene	βCAD	1469	1518
δ-Cadinene	δCAD	1469	1524
Germacrene B	GB	1486	1557
1,3-Di-tertiary-butylbenzene	DTB	1259	1281
(Z)-3-Hexenyl Acetate	ZHAC	1012	1005
Eugenol	EUG	1365	1357

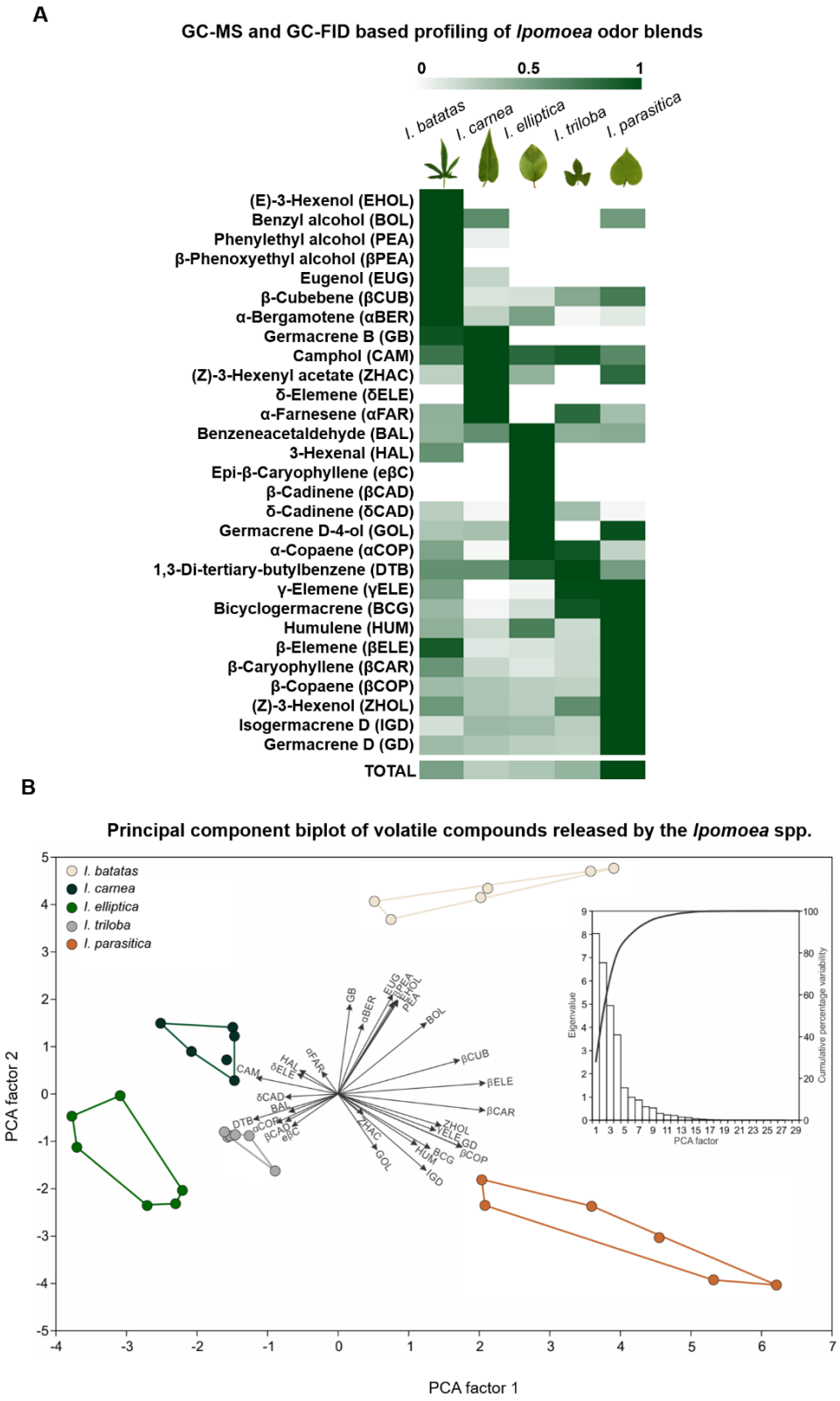

Figure 2.5: The five *Ipomoea* spp. release characteristic odor blends. (**A**) The five *Ipomoea* spp. are associated with distinct compositions of volatile compounds (Table 1). Columns in the heatmap represent *Ipomoea* spp. and rows represent compounds. Each cell represents mean concentration of a compound (nmol/ g), relative to nonyl acetate internal standard. Values in each row are normalized to the highest value in that row (n= 6). (**B**) Principal component biplot of factor scores of plant species and factor loadings

of volatiles. Scree plot of eigenvalue and percentage variation explained by each PCA factor is provided in the inset. Pairwise comparisons of clusters using ANOSIM based on Manhattan distances showed that all clusters are significantly different from each other (Table 3).

Table 3: Pairwise comparisons of clusters using ANOSIM based on Manhattan distances. R values are provided above diagonal and associated sequential Bonferroni corrected P values are provided below the diagonal. All clusters are significantly different from each other.

	I. batatas	*I. carnea*	*I. elliptica*	*I. triloba*	*I. parasitica*
I. batatas		0.8944	0.7630	0.9389	0.7741
I. carnea	0.0018		0.3907	0.9907	0.9444
I. elliptica	0.0027	0.0128		0.7000	0.9574
I. triloba	0.0019	0.0023	0.0019		0.8963
I. parasitica	0.0031	0.0020	0.0028	0.0011	

2.3.6 Hostplant preferences of *Chiridopsis* spp. are associated with specific plant VOCs

PLS analysis helped visualize the relationships between the 29 VOCs, five *Ipomoea* spp. and four *Chiridopsis* spp. by plotting them as vectors (Fig. 2.6). The angle between two vectors indicates their relation to each other. The monophagous *C. nigropunctata* and biphagous *C. undecimnotata* showed similar VOCs associated with their feeding behavior. Both their preferences strongly correlated to the first component of PLS, which also positively correlated with compounds such as β-cadinene, 3-hexenal, epi-β-caryophyllene, benzeneacetaldehyde and α-copaene, and negatively correlated with β-caryophyllene, α-farnesene, γ-elemene, benzyl alcohol, β-elemene and (*Z*)-hex-3-en-1-ol (Fig. 2.6). The similarity in plant VOCs they correlate to could explain their common preference of *I. elliptica* as hostplant. Although *C. bistrimaculata* and *C. bipunctata* have the same hostplants, their feeding preferences correlated oppositely. *C. bistrimaculata* negatively correlated, whereas *C. bipunctata* positively correlated with the second component of PLS, to which α-bergamotene, β-phenoxyethyl alcohol, (*E*)-hex-3-en-1-ol, phenylethyl alcohol, eugenol, β-cubebene, and β-elemene positively correlated, and δ-elemene, α-farnesene, and (*Z*)-3-hexenyl acetate negatively correlated (Fig. 2.6). Therefore, despite sharing a host range, these two beetles associate with different VOCs, resulting in opposite relationships.

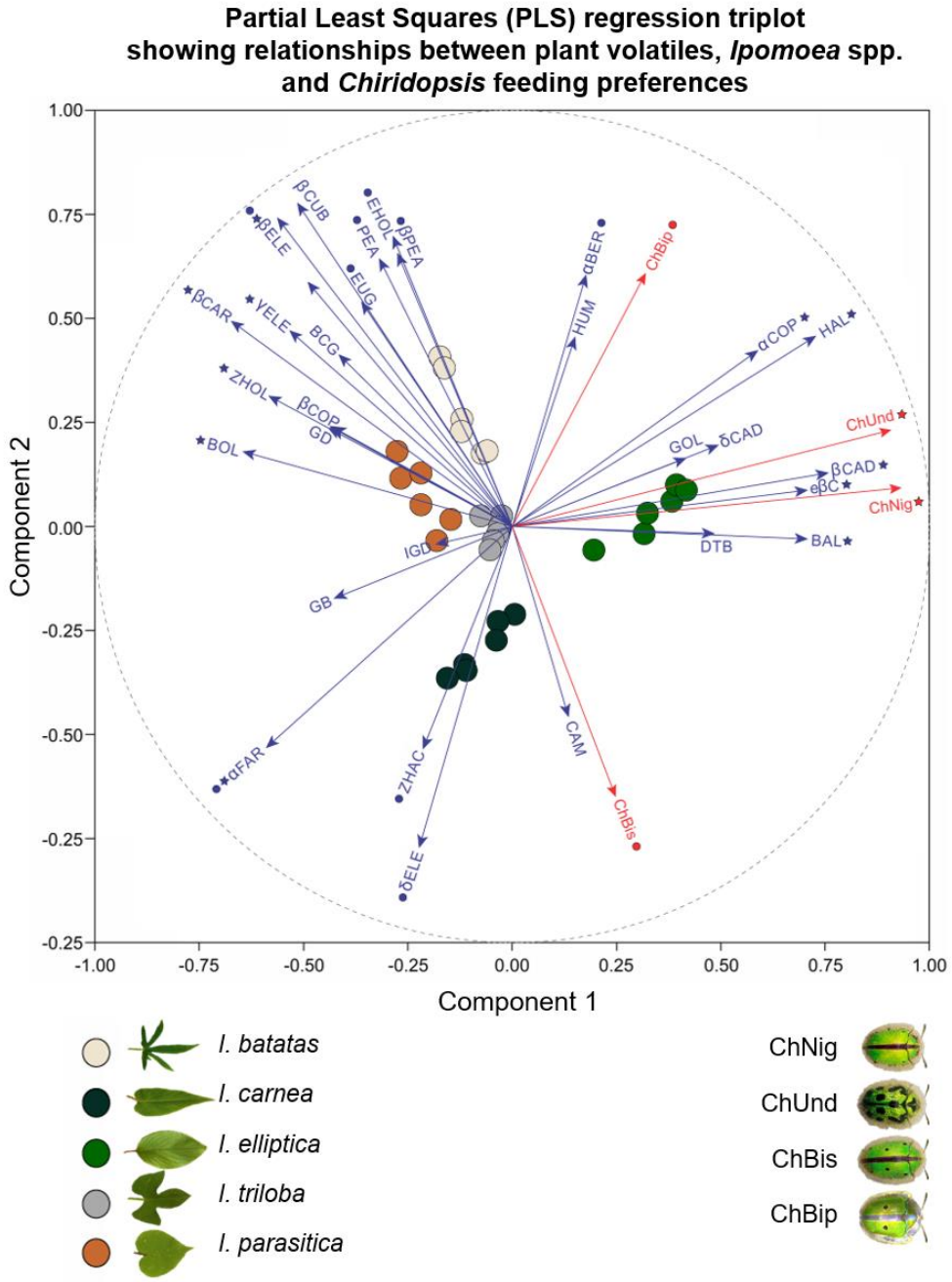

Figure 2.6: Understanding the relationships between four *Chiridopsis* feeding preferences and five *Ipomoea* odor blends. The partial least squares (PLS) regression triplot shows factor scores of *Ipomoea* spp., factor loadings of volatiles and factor loadings of *Chiridopsis* feeding preference as estimated by multiple choice assays. Independent variables (volatiles) are shown as blue vectors, while dependent variables (feeding preferences) are shown as red vectors. Correlations are absolute on the dashed unit circle. Filled star with the name of the variable indicates that the variable is significantly correlated on the first PLS axis, filled circles indicate that the variable is significantly correlated on the second PLS axis, while both stars and circles indicate that variables are significantly correlated on both the axes. Significance is assessed after sequential Bonferroni correction.

Multiple regression between the *Ipomoea* VOCs and *Chiridopsis* feeding preferences and analysis of standardized coefficients showed several significant relationships (Fig. 2.7, 2.8A). *C. nigropunctata* and *C. undecimnotata* significantly correlated with the same VOCs; positively with 3-hexenal, benzeneacetaldehyde and β-cadinene, epi-β-caryophyllene, and germacrene-D-4-ol, and negatively with (*Z*)-3-hex-3-en-1-ol, benzyl alcohol, γ-elemene, δ-elemene, bicyclogermacrene, α-farnesene and germacrene-B (Fig. 2.7A, B, 2.8A). Additionally, *C. nigropunctata* negatively correlated with phenylethyl alcohol, β-cubebene, and β-elemene, while *C. undecimnotata* did not. *C. bistrimaculata* and *C. bipunctata* correlated oppositely to some volatiles, such as δ-elemene, (*E*)-3-hex-3-en-1-ol and β-phenoxyethyl alcohol (Fig. 2.7C, D, 2.8A). For each *Chiridopsis* spp., we categorized the positively and negatively correlated plant volatiles as putative attractants and repellents, respectively.

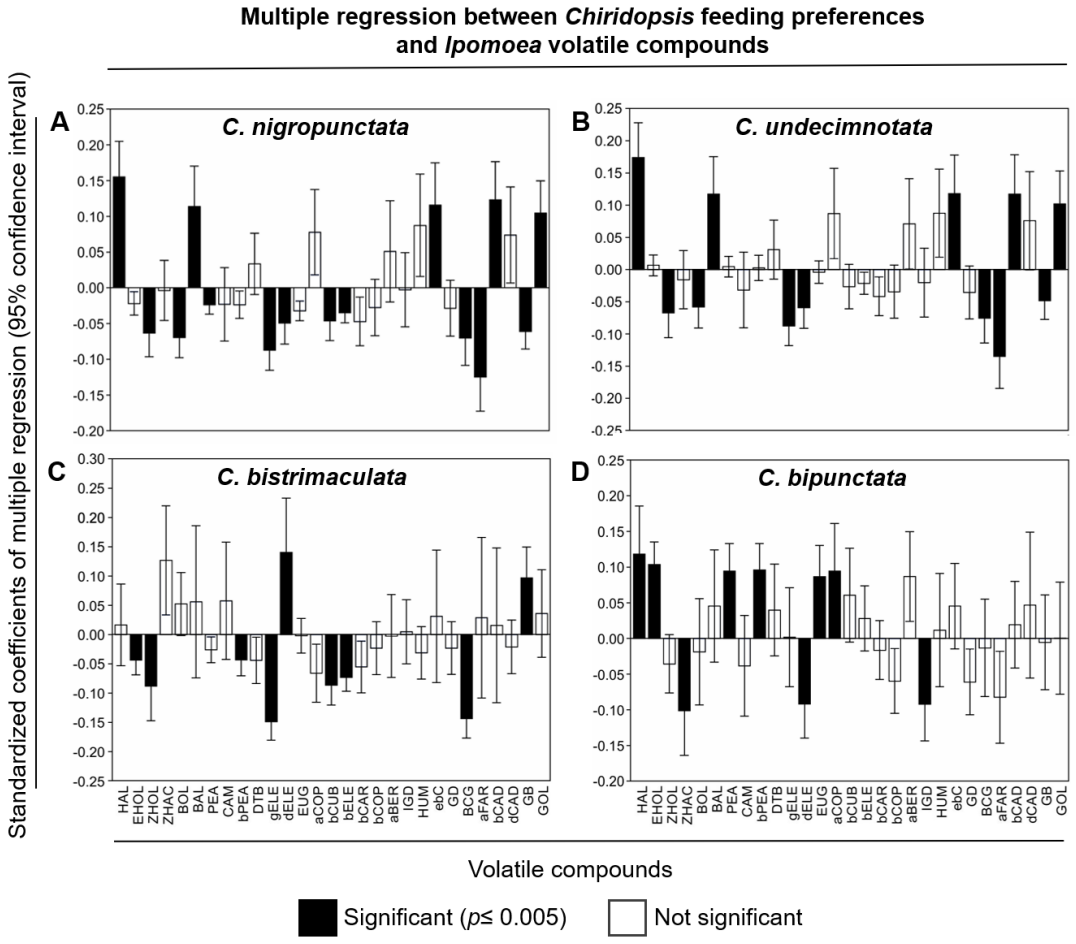

Figure 2.7: Host preference of each *Chiridopsis* spp. correlates with certain volatile compounds in *Ipomoea* odor blends. (**A**) to (**D**) Standardized coefficients of multiple regression between feeding preference and plant volatiles. Dependent variable feeding preference of (**A**) *C. nigropunctata*, (**B**) *C. undecimnotata*, (**C**) *C. bistrimaculata* and (**D**) *C. bipunctata*. Error bars are 95% confidence intervals. Bars in black are significant after sequential Bonferroni correction (p≤ 0.005).

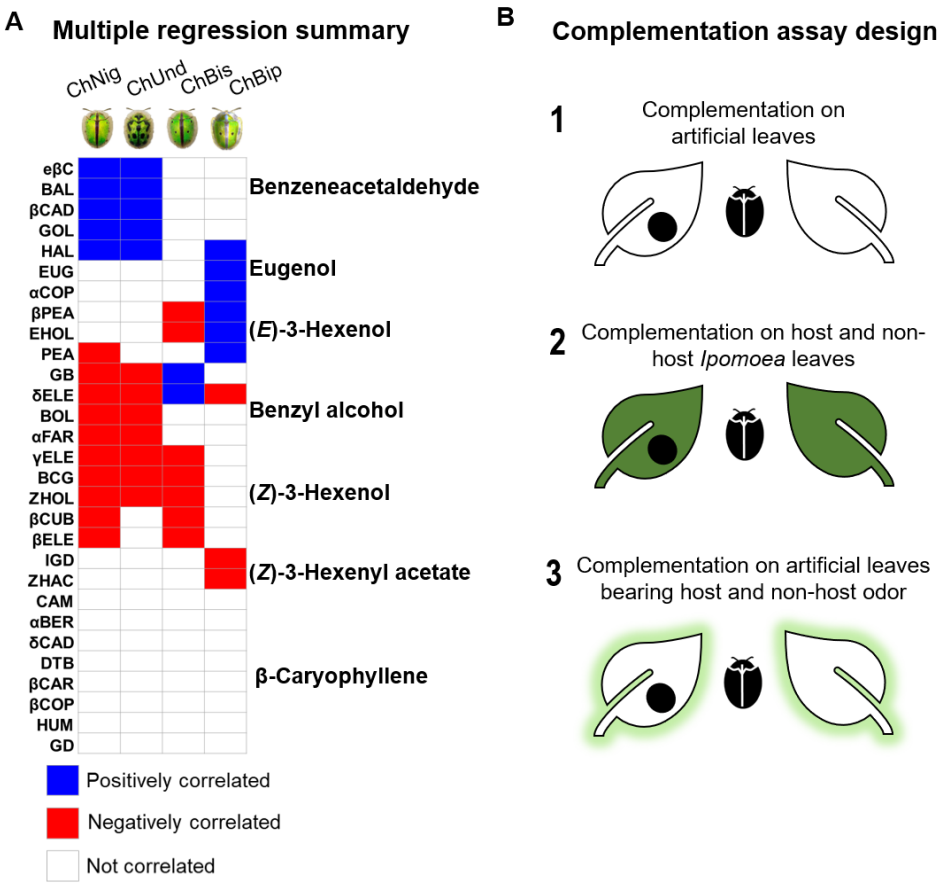

Figure 2.8: Putative attractants and repellents were predicted and tested experimentally. (**A**) Summary of multiple regression between *Chiridopsis* feeding preferences and *Ipomoea* volatiles, with compounds that show positive (blue), negative (red) and no (white) correlations. These compounds were considered putative attractants, putative repellents, and neutral compounds respectively. (**B**) Candidate compounds were tested experimentally by complementing pure compounds on artificial leaves, host and non-host leaves, and artificial leaves bearing host or non-host odor. Beetles were then subjected to dual choice assays between candidate compound-complemented and solvent-complemented leaves.

2.3.7 *Chiridopsis* spp. respond to attractant and repellent volatiles only when presented within a hostplant's odor

We tried to experimentally test the functions of the candidate volatiles (putative attractants and repellents) through a series of complementation assays (Fig. 2.8B). Presenting individual candidate compounds in serially increasing concentrations neither attracted nor deterred beetles (Fig. 2.9 A, C, E, G). On the contrary, when their concentration was serially increased on each beetle's two most-preferred plants and two least-preferred plants, the compounds were indeed of attractant or repellent nature as predicted by the multiple regression (Fig. 2.10, 2.11, 2.12, 2.13). At each concentration of an attractant on a hostplant, most beetles preferred the complemented leaf (Fig. 2.10,

2.11). Moreover, with increasing concentration of pasted attractant, complemented leaves were preferred by an increasing number of beetles. Conversely, when we complemented repellents on hostplants, most beetles preferred the non-complemented leaf. As we increased repellent concentration, fewer beetles preferred the complemented hostplant leaf. We observed this attraction/ deterrence behavior only when we pasted attractants/ repellents on the beetles' natural hostplants. On non-host leaves, beetles' behavior remained unaffected. The beetles did not exhibit this increased attraction/ deterrence upon increasing concentration of compounds that did not correlate with host preference (neutral) or compounds that had not been detected in the *Ipomoea* spp. (foreign) (Fig. 2.12, 2.13), thus empirically validating the multiple regression results. These observations also held when the candidate compounds were pasted on artificial leaves pre-coated with hostplant odor blends (Fig. 2.14), showing that the behavioral responses observed were due to olfactory signals alone.

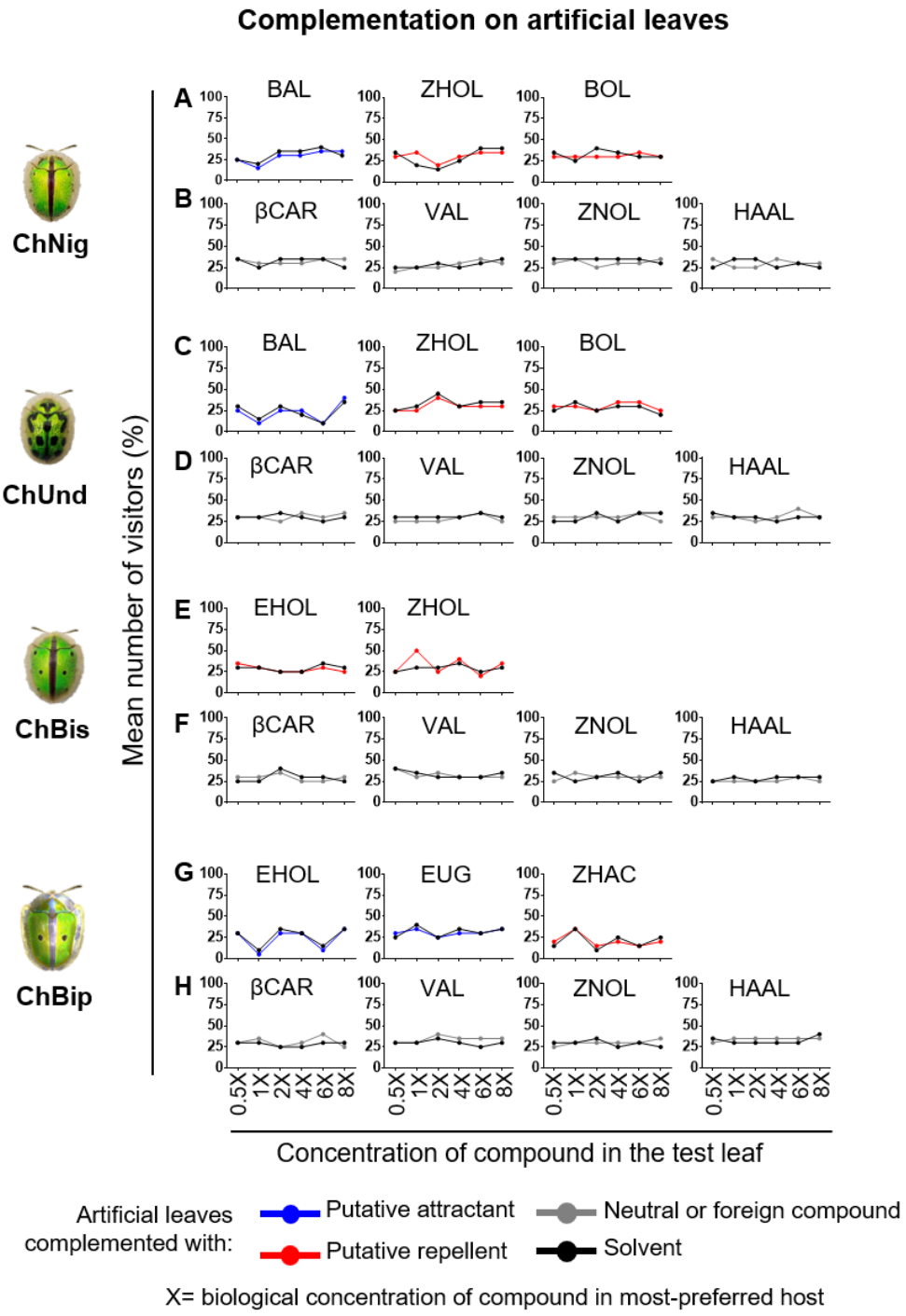

Figure 2.9: *Chiridopsis* spp. do not respond to volatile compounds presented singly. Putative attractants and putative repellents (**A, C, E, G**) for *C. nigropunctata*, *C. undecimnotata*, *C. bistrimaculata* and *C. bipunctata* were individually complemented on artificial leaves in increasing concentrations. In addition to putative attractants and repellents, assays were also performed using the following technical controls: compound detected in *Ipomoea* spp. but not correlated to beetles' preferences (neutral compound: β-Caryophyllene) and compound not detected in any of the five *Ipomoea* spp. in study (foreign compounds) (**B, D, F, H**). For the latter, a green leaf volatile (hexanal), an aliphatic alcohol ((*Z*)-3-nonen-1-ol) and a sesquiterpene (valencene) were used. Since these compounds were not detected in the *Ipomoea* spp., the reported biological concentration in their close relatives was considered as 1X (see Materials and Methods section). Beetles were subjected to a dual choice assay between solvent-pasted (control) and compound-pasted (test) artificial leaves. Behavioral response to the test compounds was estimated as number of visitors to each leaf. Data shown in the figure is mean percentage of visitors on control and test leaves (n= 20). In all cases, beetles visited both control and complemented leaves similarly, showing no significant preference.

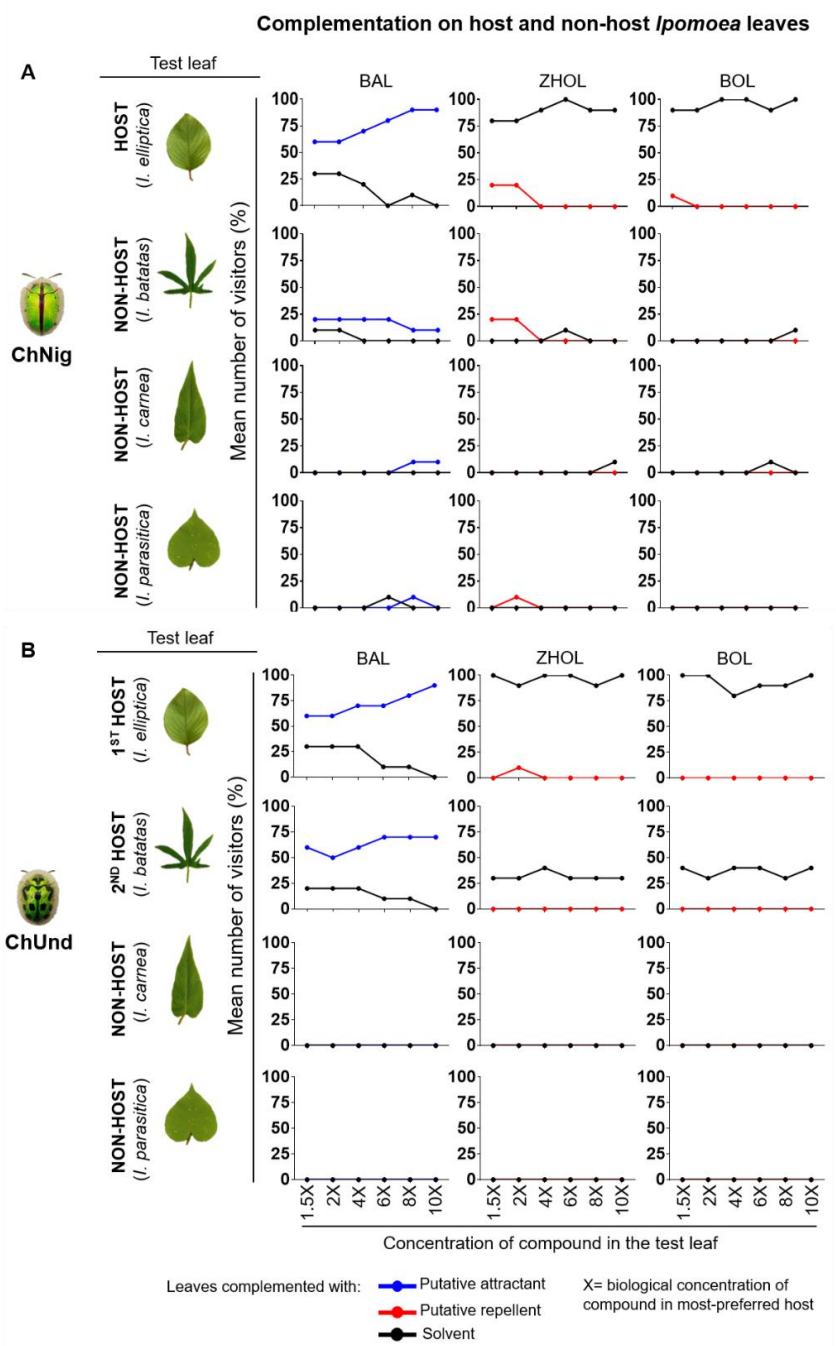

Figure 2.10: *Chiridopsis* spp. respond to attractant and repellent volatiles only when encountered along with their hostplant's odor blend. On leaves of each *Chiridopsis* sp.'s two most preferred and two least preferred plants, we serially raised the concentration of putative attractants and repellents. Beetles were subjected to dual choice assays between leaves pasted with a putative attractant/ repellent (test) and leaves pasted with only solvent (control). Preference of each *Chiridopsis* sp. was analysed by quantifying the number of beetles who visited each choice. Data shown in the figure is mean percentage of visitors on control and test leaves (n= 10) for (**A**) *C. nigropunctata* and (**B**) *C. undecimnotata*. Contrary to when these compounds were presented on their own (Fig. 2.9), beetles exhibited behavioural attraction or deterrence when these compounds were presented on leaves naturally releasing their respective odor blends. As concentration of each attractant/ repellent was incremented, correspondingly more/ less beetles visited the leaf. This behavioural response was displayed only when the test leaf was of a natural host. In addition to putative attractants and repellents, as

technical controls we also serially raised the concentration of neutral and foreign compounds. In these cases, no preference was shown by beetles (Fig. 2.12).

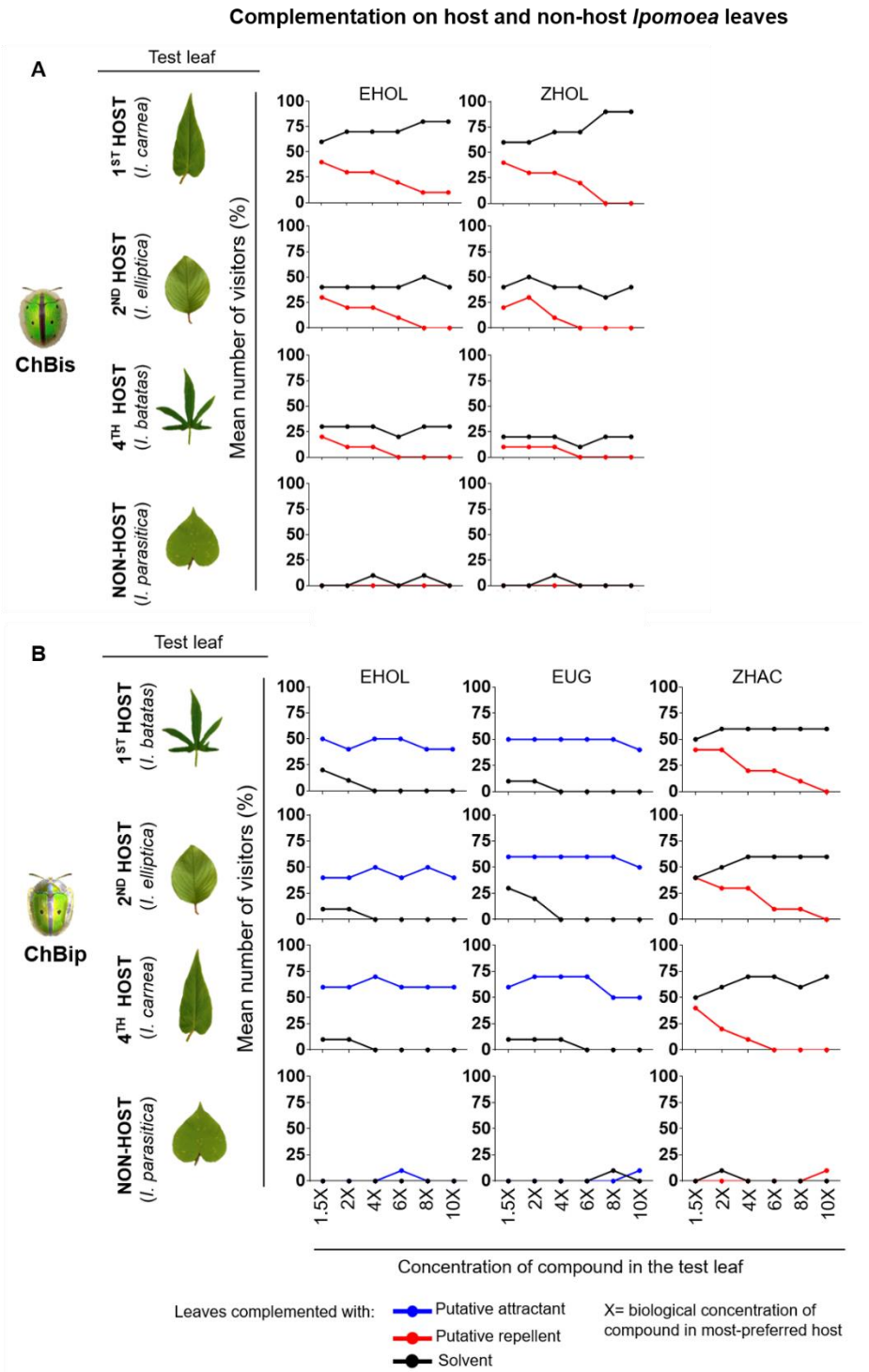

Figure 2.11: *Chiridopsis* spp. respond to attractant and repellent volatiles only when encountered along with their hostplant's odor blend. (**A**) *C. bistrimaculata* and (**B**) *C. bipunctata* beetles also displayed attraction and repellence when the candidate compounds were presented on leaves. Preference was analysed by quantifying the number of beetles who visited each choice. Data shown in the figure is mean percentage of visitors on control and test leaves (n= 10). As concentration of each attractant/ repellent was incremented, correspondingly more/ less beetles visited the leaf. This behavioural response was displayed only when the test leaf was of a natural host. In addition to putative attractants and repellents, as technical controls we also serially raised the concentration of neutral and foreign compounds. In these cases, no preference was shown by beetles (Fig. 2.13).

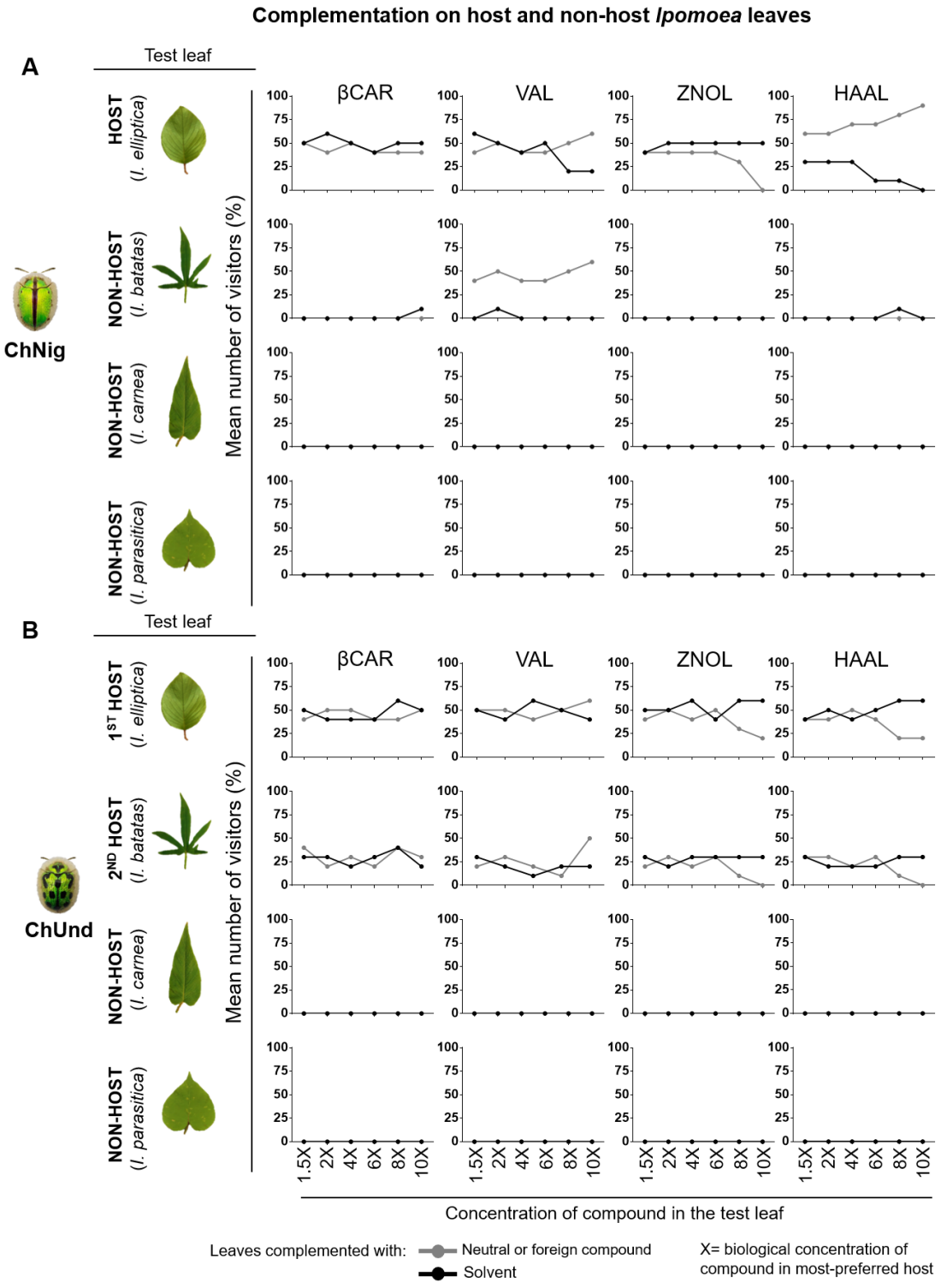

Figure 2.12: *Chiridopsis* spp. do not respond to volatiles that are uncorrelated to their feeding preferences or those not detected in the *Ipomoea* spp. In addition to putative attractants and repellents (Fig. 2.10. 2.11), as technical controls we also serially raised the concentration of the following: compound not correlated with beetle feeding (neutral compound: βCAR) and compounds not detected in the *Ipomoea* spp. (VAL, ZNOL, HAAL). Beetles were subjected to dual choice assays between leaves pasted with compound (test) and leaves pasted with only solvent (control). Beetle preference was analysed by quantifying the number of beetles who visited each choice. Data shown in the figure is mean percentage of visitors on control and test leaves for (**A**) *C. nigropunctata* and (**B**) *C. undecimnotata* (n= 10).

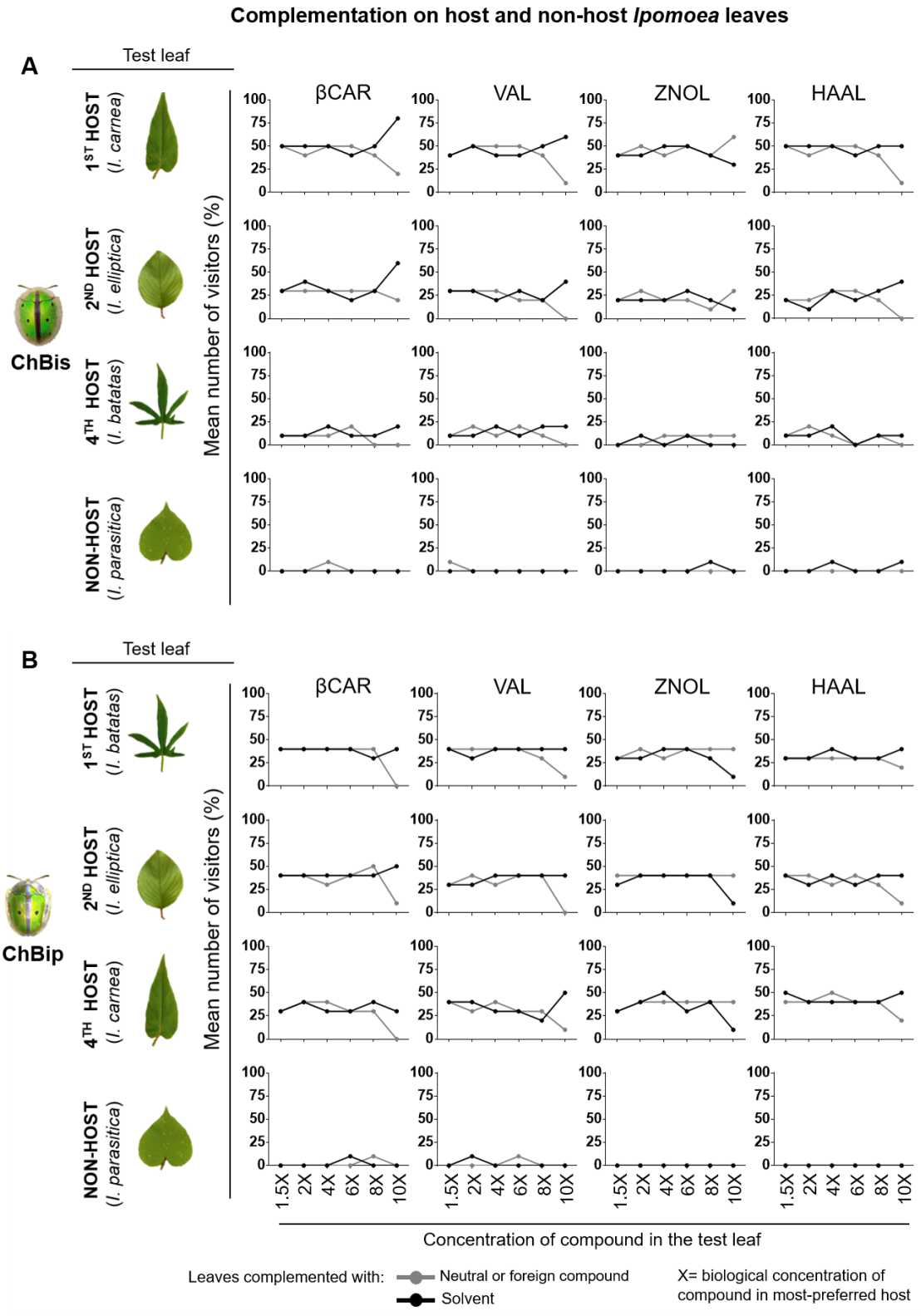

Figure 2.13: Chiridopsis spp. do not respond to volatiles that are uncorrelated to their feeding preferences or those not detected in the *Ipomoea* spp. When exposed to serially increasing concentrations of neutral and foreign compounds, (**A**) *C. bistrimaculata* and (**B**) *C. bipunctata* beetles did not show any preference. Beetle preference was analysed by quantifying the number of beetles who visited solvent-pasted (control) and compound-pasted (test) each choice. Data shown in the figure is mean percentage of visitors on control and test leaves (n= 10).

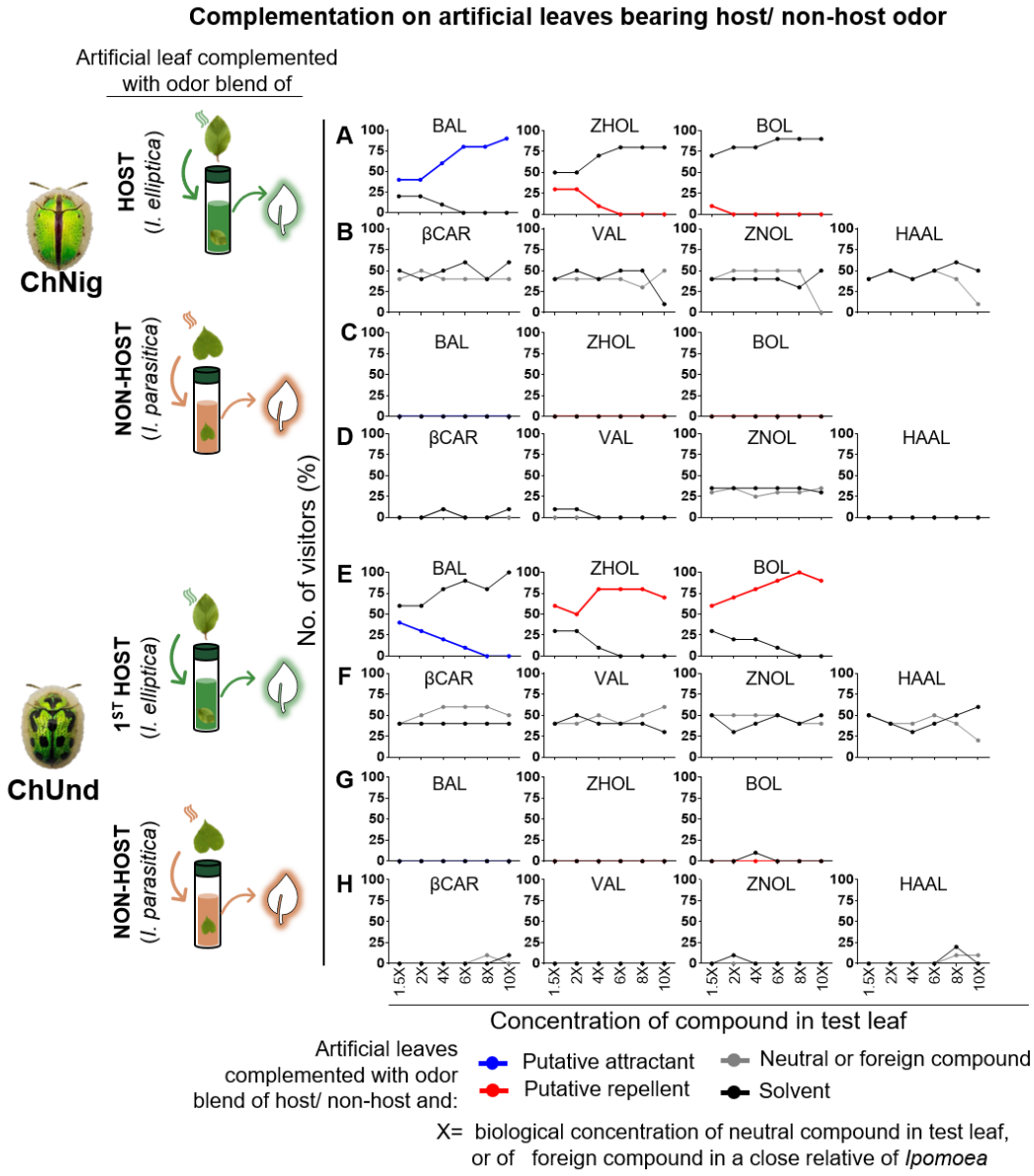

Figure 2.14: *Chiridopsis* spp. respond to attractant and repellent volatiles only when delivered through hostplant's odor blend. On artificial leaves coated with the odor blends of most-preferred hosts or non-hosts, we serially increased the concentrations of putative attractants, putative repellents, neutral or foreign compound repellents for each *Chiridopsis* sp. Beetles were subjected to dual choice assays between artificial leaves coated with only odor blend (control) and those coated with a blend+ compound (test). Preference was estimated as the number of beetles who visited each choice. Data shown in the figure is mean percentage of visitors on control and test leaves (n= 10) for (**A**) to (**D**) *C. nigropunctata* and (**E**) to (**H**) *C. undecimnotata*. Similar to when these compounds were encountered on different leaves (Fig. 2.10), all beetles exhibited behavioural attraction or avoidance when these compounds were encountered within odor blends, in a concentration-dependant manner. This behavior was displayed only when the background odor of the test leaf belonged to a natural host (**A**, **E**). If the pasted blend was of a non-host (**C**, **D**, **G**, **H**), then increasing levels of attractants/ repellents did not result in more/ less visits respectively. Beetles showed no response to neutral or foreign compounds when provided through host or non-host odor blends. Together, these results indicate that attractants and repellents exert their function only when present within a hostplant's odor blend.

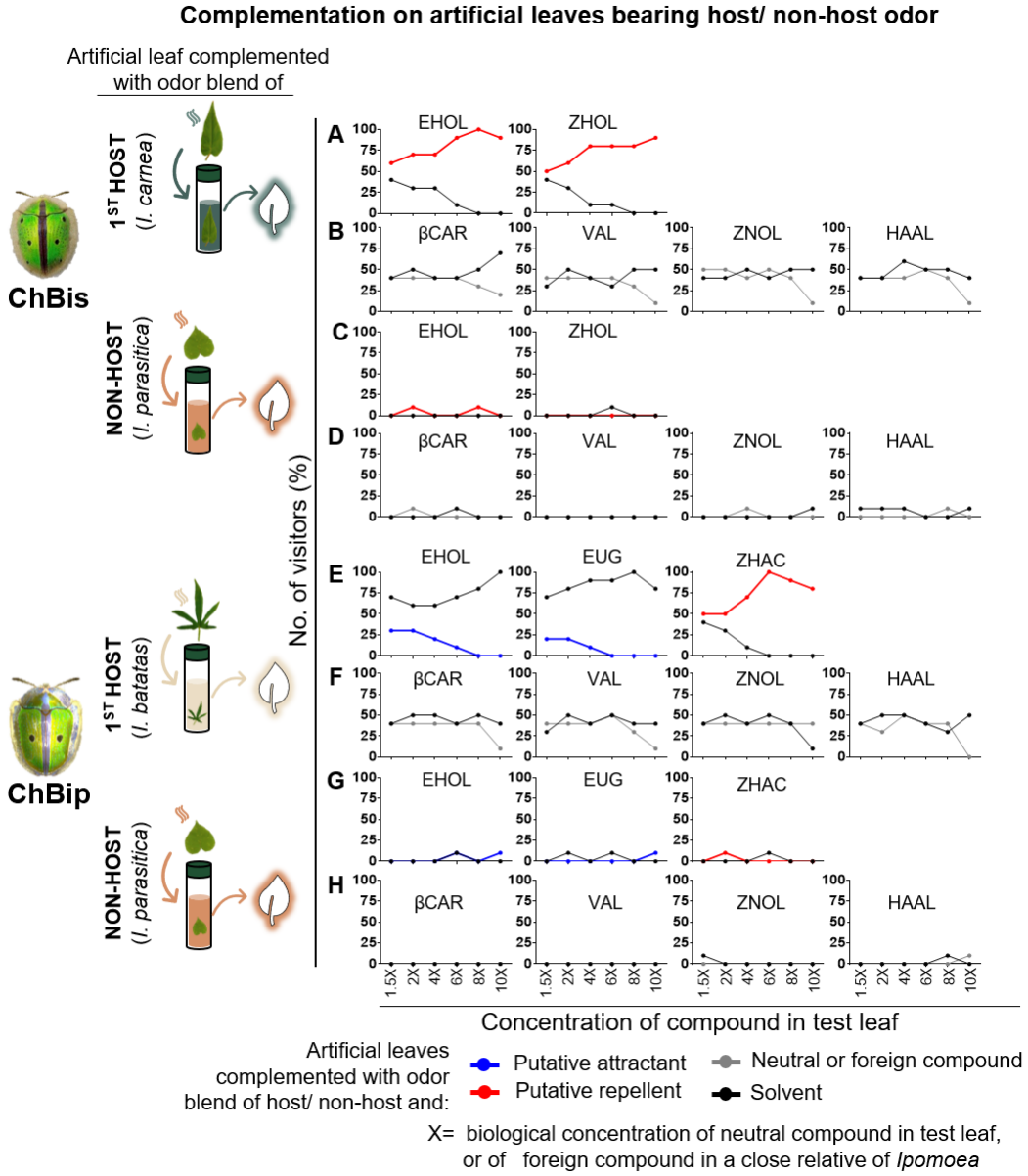

Figure 2.15: *Chiridopsis* spp. respond to attractant and repellent volatiles only when delivered through hostplant's odor blend. On artificial leaves coated with the odor blends of most-preferred hosts or non-hosts, *C. bistrimaculata* and *C. bipunctata* showed similar behavior as *C. nigropunctata* and *C. undecimnotata* (Fig. 2.14). Data shown in the figure is mean percentage of visitors on control and test leaves (n= 10) for (**A**) to (**D**) *C. bistrimaculata* and (**E**) to (**H**) *C. bipunctata*. Similar to when these compounds were encountered on different leaves (Fig. 2.11), all beetles exhibited behavioural attraction or avoidance when these compounds were encountered within odor blends, in a concentration-dependant manner. This was observed only when the background odor of the test leaf belonged to a natural host (**A**, **E**). If the pasted blend was of a non-host (**C**, **D**, **G**, **H**), then increasing levels of attractants/ repellents did not result in more/ less visits respectively. Beetles showed no response to neutral or foreign compounds when provided through host or non-host odor blends.

2.3.8 Candidate attractants, repellents and neutrals are present in the headspace

Detection of the experimentally tested VOCs in the *Ipomoea* headspace (Fig. 2.16) by SPME-HS analysis ascertained that the compounds are indeed released by plants into the environment.

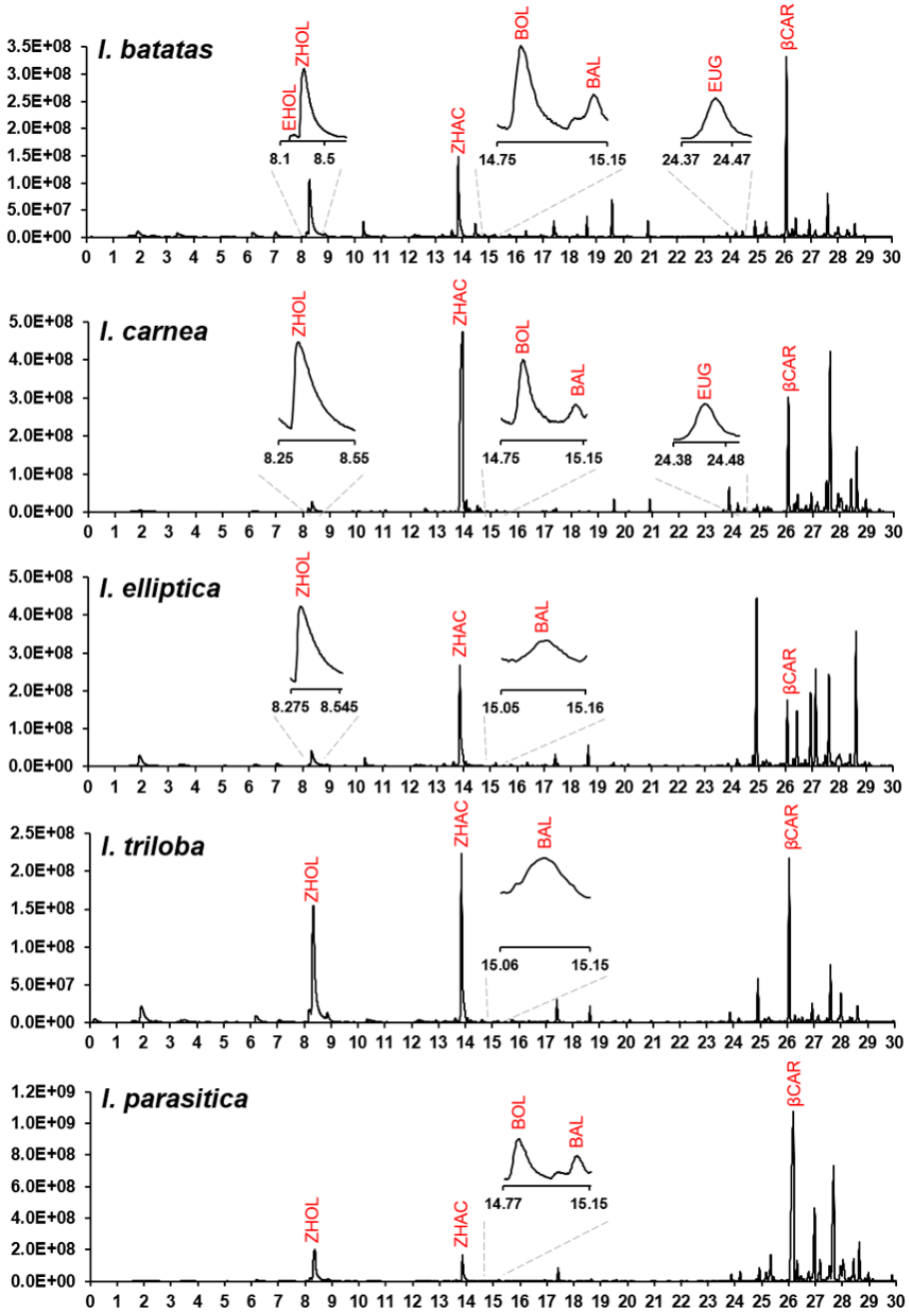

Figure 2.16: Experimentally verified attractants, repellents, and neutrals are present in *Ipomoea* headspace. For each species, a potted plant was enclosed in a ventilated glass cylinder, and exposed to an SPME fiber assembly (divinylbenzene/ carboxen/ polydimethylsiloxane) for 1 h to collect headspace volatiles. Headspace volatiles detected are shown in GC-MS chromatograms for each *Ipomoea* sp. All experimentally tested compounds were detected in the headspace odor.

2.3.9 Experimentally validated test compounds are EAG-active

EAG analysis showed that all the attractant, repellent, and neutral test compounds elicited electrophysiological response in beetles' antennae, suggesting that they are perceived by olfactory receptors. Increased responses were observed with corresponding increase in stimulus concentration, with saturation beyond a threshold concentration in some cases (Fig. 2.17).

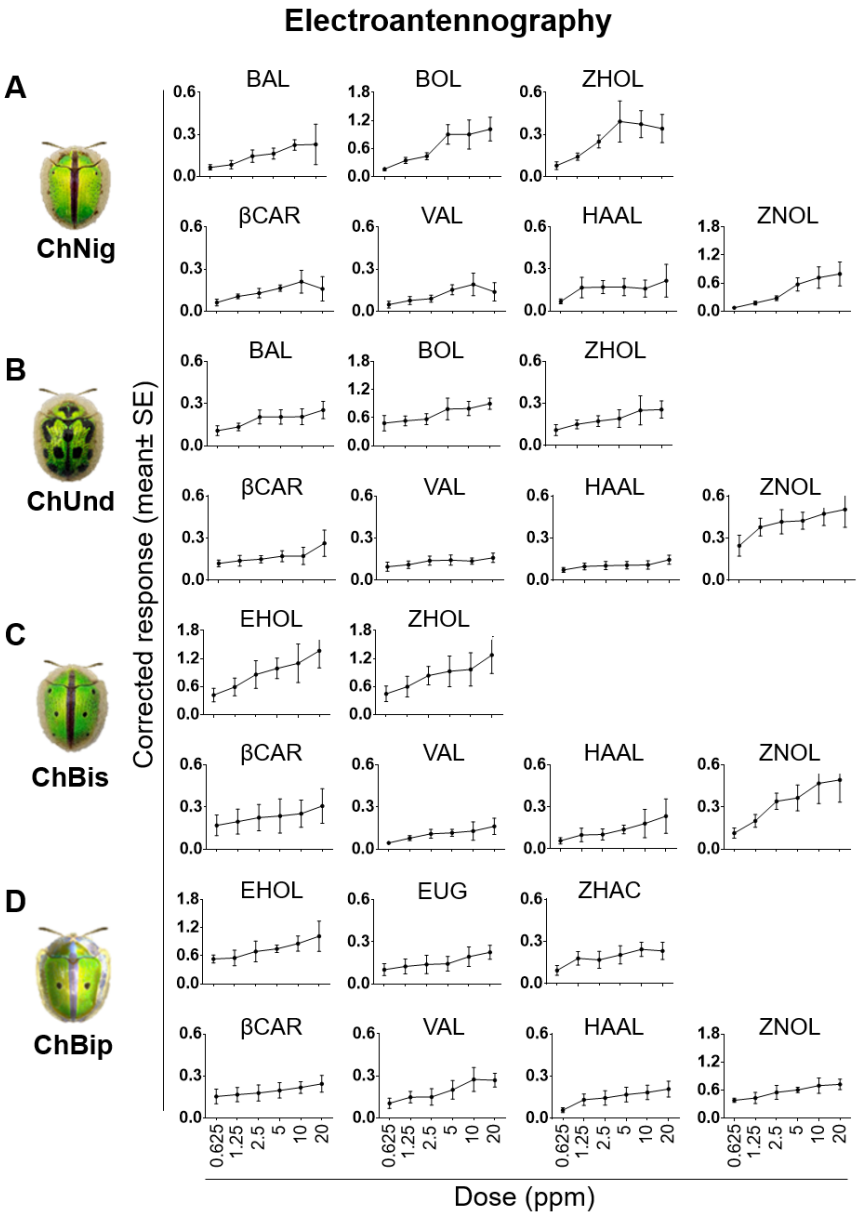

Figure 2.17: Experimentally tested attractants, repellents and neutrals are EAG-active. Electrophysiological response was observed in antennae of (**A**) *C. nigropunctata*, (**B**) *C. undecimnotata*, (**C**) *C. bistrimaculata*, and (**D**) *C. bipunctata* towards all the experimental candidate compounds, suggesting that they have olfactory receptors for all the compounds. Increasing EAG response was observed with increasing doses of compounds. Data shown in the figure is the blank subtracted response. Blank in all cases was DCM.

2.3.10 Odor imaging

The proportions of attractants, repellents, and neutrals in each *Ipomoea* sp. was plotted as a pie diagram for all the *Chiridopsis* sp. (Fig. 2.18). Odor images generated from these proportions for all beetle and plant combinations revealed that the beetles perceive the five plant odors differently, visualized as color differences (Fig. 2.19). For all beetles, the first host appeared with an attractant blue hue, due to the higher proportion of attractants (Fig. 2.19C, H, L, P, R). The hues of green and red were seen in odor images of less-preferred hostplants or non-hosts, due to their higher proportions of neutral and repellent compounds. The odor images also reveal that different beetles perceive the same hostplant using different attractants, repellents, and neutrals, and thus perceive the sane plant with different odor images (Fig. 2.19A-P, B-Q, C-R, D-S, E-T).

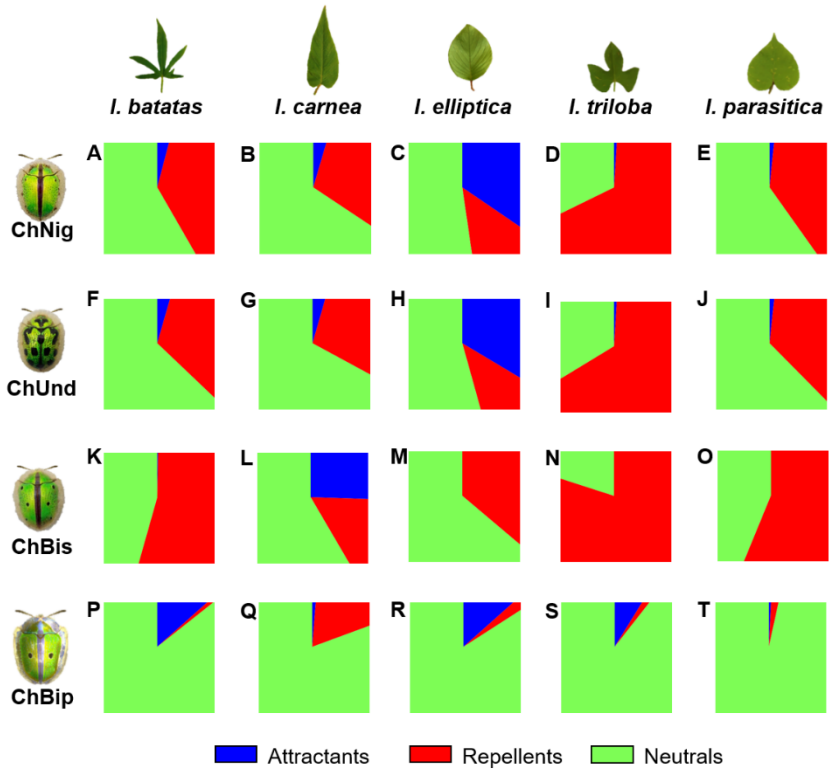

Figure 2.18: Each *Ipomoea* sp.'s odor blend has a signature proportion of attractants, repellents, and neutrals, which varies for different *Chiridopsis* sp. For every *Chiridopsis* sp., the concentration of each attractant (blue), repellent (red), and neutral compound (green) (Table 1) was multiplied by the beetle's standardized regression coefficient for that compound. Resulting values were plotted as a pie diagram for (**A**) to (**E**) *C. nigropunctata*, (**F**) to (**J**) *C. undecimnotata*, (**K**) to (**O**) *C. bistrimaculata* and (**P**) to (**T**) *C. bipunctata*, resulting in a different pie diagram for each beetle-plant pair. Pie diagrams were plotted as squares instead of circles for ease of using them to pixelate leaf shapes (Fig. 2.19).

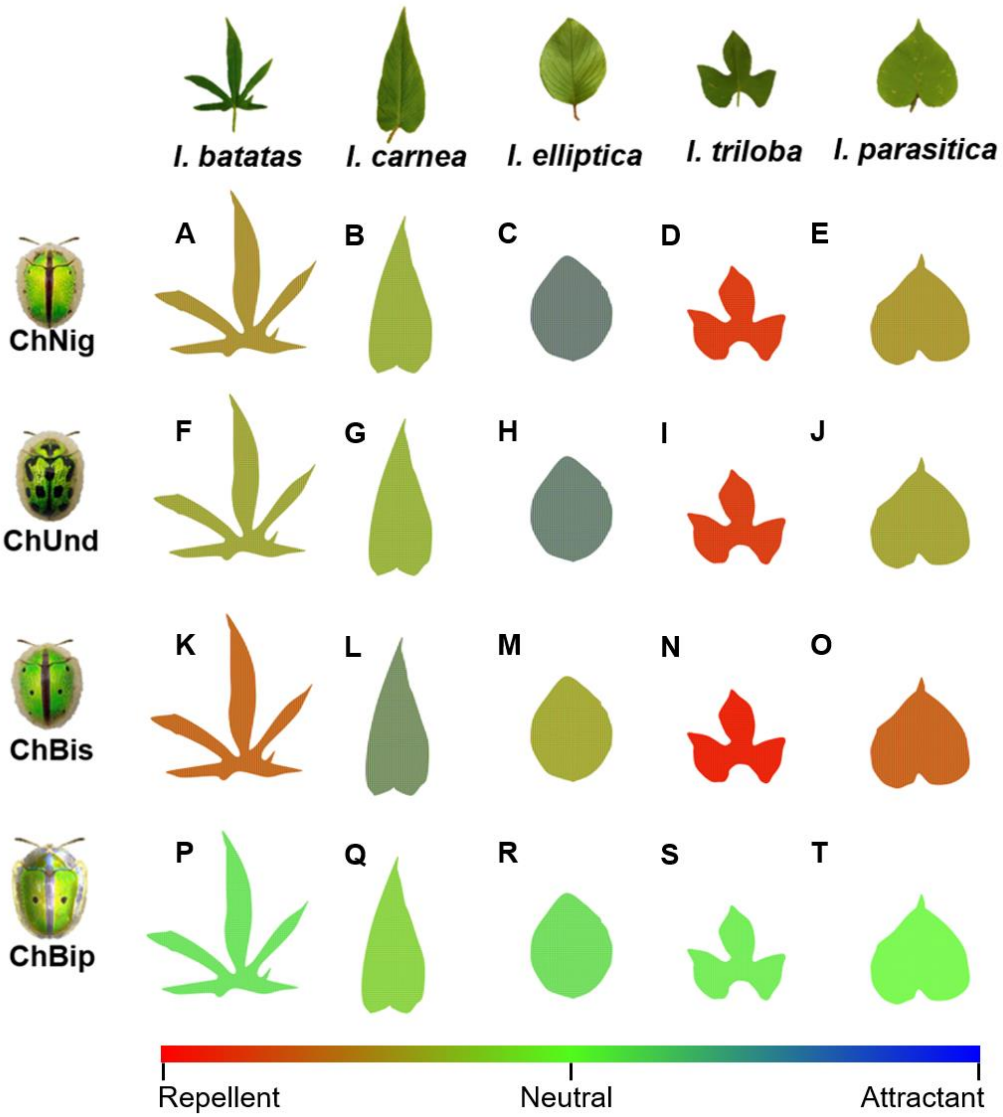

Figure 2.19: Each *Ipomoea* spp. is associated with a different characteristic odor image for each *Chiridopsis* spp. For every beetle-plant pair, the pie diagrams in Fig. 2.18 were used to pixelate respective leaf shapes, thereby causing all 20 leaves to gain different hues. The odor images show that each beetle perceives the five *Ipomoea* spp. differently (**A**) to (**E**), (**F**) to (**J**), (**K**) to (**O**), (**P**) to (**T**). Leaves of most-preferred hosts appear with a blue hue due to higher proportion of attractants (**C**, **H**, **L**, **P**, **R**), whereas those of less-preferred hosts (**F, K, N, Q, S**) or non-hosts (**A, B, D, E, G, I, J, O, T**) appear with a yellow or red hue due to higher proportion of neutrals or repellents. When odors are similar, their resolution is based on the proportions of attractants and repellents. The odor images also show how the same plant is perceived with different odor images by different beetles, depending on their preferences. Through this novel odor imaging tool, we attempted to create a visual representation of beetles' in-flight perceptions of host and non-host odors.

2.4 Discussion

In the *Ipomoea-Chiridopsis* interaction, the host preference spectrum of one insect genus is exclusively associated with different species of only one genus of hostplants. In this naturally sympatric system, the insects show highly host-specific occurrences, which they maintain through every life cycle stage. Consistent with these field observations, we observed a striking trend in the hostplant preferences displayed by these beetles in our laboratory experiments. The choice of hostplants to feed displayed the same trend as the adult and larval survivorship on the different plants. While we observed 100% survivorship of *C. nigropunctata* and *C. undecimnotata* on their most-preferred hosts, *C. bistrimaculata* and *C. bipunctata* displayed survivorship on all their hosts without 100% survival on any one host. This was not surprising as oligophagous insects often tend to have moderate viability on each host and no maximal fitness on a single host[94–96]. Together our results indicate a fine-tuned relationship between these beetles and plants, suggesting that the insect choices are evolutionary adaptations for greater survival rather than spontaneous foraging decisions.

The high specialization observed in this insect-hostplant system, especially considering that all species co-occur, led us to investigate the basis of the precise host identification. Insects are known to begin the hostplant identification process in-flight, using contactless visual and olfactory cues. Despite variable weather conditions and the low resolution of insect vision, we have observed that these beetles directly land on their hosts, suggesting that visual cues are not the major signals associated with *Chiridopsis* host location. On the other hand, the role of olfactory signals in hostplant location by insects is well recognized[4,97,98]. Our observation that hostplant odor alone was sufficient to elicit beetle visits indicated that the major host identification signal in this system is plant odor. GC-MS-FID-based profiling revealed that the *Ipomoea* species are associated with a similar set of VOCs, but the five blends differ in their proportions and concentrations of these compounds. The odor blends were rich in sesquiterpenes but no monoterpene was detected in both GC-MS/FID and SPME analyses, indicating that the *Ipomoea* spp. in this study did not produce monoterpenes. PCA and ANOSIM analysis showed that due to these quantitative differences, the five congeneric sympatric plants had significantly different odors. Understanding how the 29 plant VOCs in five *Ipomoea* spp. correlate with hostplant preferences of four *Chiridopsis* spp. required a multivariate statistical

approach to deal with the high dimensionality of the data. PLS analysis and multiple regression helped visualize the relationships between these dimensions, and revealed that some VOCs are associated with the feeding preferences of each beetle. A high resemblance exists between monophagous *C. nigropunctata* and biphagous *C. undecimnotata* in this regard. Both beetles positively and negatively correlate similarly with a group of VOCs; this explains their common choice of *I. elliptica* as the most-preferred hostplant. The only correlated VOCs differentiating these beetles (phenylethyl alcohol, β-cubebene, and β-elemene) are higher in *I. batatas* than *I. elliptica*, thus explaining their negative correlation to *C. nigropunctata* for who *I. batatas* is a non-host. Interestingly, despite sharing the same range of hostplants, oligophagous *C. bistrimaculata* and *C. bipunctata* correlate negatively with each other, and show nearly opposite trends of preferences within their four *Ipomoea* hosts. Compounds that are attractants for one are repellents for the other, and vice-versa. Their host preferences correlate with different VOCs, suggesting that these two sympatric beetles recognize the same plants using different cues.

Through a series of complementation assays, we demonstrated how the correlated compounds function as attractants or repellents, and our results agree with statistical correlations. The observation that beetles do not respond to putative attractants and repellents when presented singly, whereas they do respond to odor blends, led us to hypothesize that the attractant and repellent volatiles are functional only when they co-occur with other hostplant volatiles in a blend. Complementation assays supported our hypothesis and demonstrated that these compounds are critically associated with the background volatiles- the matrix. Compounds attracted and repelled beetles only when their levels increased within the hostplant- they did not have the same behavioral effect when increased in non-hosts. Furthermore, these assays showed that the attractive nature of an attractant is not brought about by the specific concentration found in a beetle's most-preferred hostplant. Similarly, for a repellent, it is not the specific concentration found in the non-host *I. parasitica*, which renders it deterrent. If this were the case, increasing attractants on non-hosts would have attracted beetles, and increasing repellents on hosts would have deterred them. Instead, we demonstrate that rather than the absolute concentration of an attractant or repellent in a plant, its co-occurrence with other compounds in a matrix confers it its identity as an attractant or repellent. Therefore, these

signals are functional only with the background of all the other compounds, together forming the plant's odor.

Together, our experiments led us to discover that *Chiridopsis* beetles' odor blend perception is contextual: the same compound can be of attractant, repellent, or neutral nature, depending on the odor background. This is in agreement with several other reported host identification studies, which found that background odor critically affects odor perception in insects[15,99,100]. The presence of other hostplant volatiles, even if repellent by themselves, has been found to make some compounds more attractive to insects and aid in host recognition[6]. This could be because such non-attractant, non-host, or repellent compounds function as habitat cues, providing an essential context to the insect that the attractants being perceived have indeed originated from a hostplant in nature. In some other cases, the presence of other volatiles has been found to have masking or distracting effects, making key attractant compounds less effective[19].

The general principles underlying the collective perception of attractants and repellents for hostplant identification have remained poorly understood[19]. We attempted to visualize this olfactory perception by odor imaging. An *Ipomoea* sp.'s odor image is formed by two components: the composition of VOCs in that plant and how each VOC affects beetle preference. While the first component distinguishes the five *Ipomoea* spp. from each other, the second distinguishes how the four *Chiridopsis* spp. perceive the same *Ipomoea* sp. Each odor image is a visual representation of a particular beetle's olfactory perception of a particular plant. Together, they suggested that within the host odor blend, the concentration of attractants and repellents is instrumental. If two odors are similar, their differentiation of them is based on the proportions of attractants and repellents. Our use of multiple insect herbivores sharing a hostplant range provided additional insight. We see that the olfactory cues are beetle-specific; the identity of attractant, repellent, and neutral compounds in the same hostplant is different for each beetle. As a result, the same plant is perceived as a different odor image by different beetles; different beetle species have evolved different behavioral responses upon perception of the same odorant from a shared host. The integration of behavior, statistics, and metabolomics to image odor perception is the first effort of its kind in the field. Odor imaging sheds light on how a flying beetle perceives its hostplant odor and distinguishes between odor blends of closely related plant species, especially when these plants occur together. These results

indicate that olfactory cues are one of the major factors associated with hostplant recognition and specialization. In the future, this tool could be fine-tuned with the incorporation of odor detection thresholds, volatility, and VOC emission rates.

Host identification by odor perception has been studied over decades in several insect systems. Studies suggest that insects either use individual compounds (attractant or repellent) or their mixtures in specific ratios to identify hostplants. Our findings are more in agreement with the latter. Through our study we demonstrate that VOCs are not independent components to study in isolation; instead, more multivariate studies on entire blends are required to understand how they may function collectively. We have attempted such a multidimensional approach to understanding plant odorscapes, and this is the first study of this kind. Such studies could provide insights into this research area as it offers a realistic perspective on understanding host/ non-host recognition by a foraging insect.

Chapter 3

Deciphering the signals that mediate hostplant-specific aggregation of *Chiridopsis* spp.

3. Deciphering the signals that mediate hostplant-specific aggregation of *Chiridopsis spp.*

3.1 Introduction

In the animal kingdom, foraging and living in groups is a widespread behavior[101]. Insects, in particular, display grouping behaviors where a large number of conspecific individuals gather to feed and breed at the food source. In 'eusocial' insect species, the aggregating conspecifics exhibit stable and organized social structures with distribution of labor, such as honey bees and ants[102,103]. Such systems have been well explored in literature[25]. Non-eusocial insect species also sometimes display aggregations, but they are temporary, for instance, while feeding. These insects are conventionally referred to as gregarious and distinguished from eusocial insects, as they do not show the characteristic social structures seen in the latter[104].

Gathering on the food source offers benefits in many forms, which have been studied in terms of fitness, benefits, and costs. Some benefits of group feeding include the facilitation of mate finding, the coordinated overcoming of plant defense[23], and better resource exploitation due to increased foraging efficiency[105–107]. A classic example is the bark beetles, where communal attack on healthy host trees helps overcome the plants' defense, ultimately killing the tree and rendering it suitable for feeding[23,108,109]. Group assemblies have also been associated with reduced predation risk, and this is sometimes related to the spatial distribution of individuals within groups[110,111]. For instance, individual group members may experience a decreased predation risk in larger groups, known as a dilution effect[112]. In some other instances of 'selfish herds,' group members located at the center of groups are at less risk of predation than those at margins[110,112,113]. Protection from predators in conspecific assemblies has also been attributed to active group defense such as release of repellents[113,114], increased vigilance[115], or aposematic aggregregations[116,117]. Some insect species even aggregate only with selected conspecific individuals to create social niches[104,118]. Such behavior has been reported to minimize conflict, aggression, and the costs associated with unfamiliar conspecifics. Altogether, the positive relationship between some aspect of the participating individual's fitness and the population number or density, has been referred to as the 'Allee effect.'[119] However, as is for most traits, the benefits of aggregation are also associated with some costs. Some

examples include increased intraspecific competition[108,120,121], the easier spread of parasites[122], and in some cases, higher attraction of predators by eavesdropping on prey aggregation signals[110,123–125].

Conspecific aggregation has been surmised to be a strategy of competitor coexistence that does not involve resource partitioning; in areas of patchy food resources distribution, spatial clustering of conspecifics decreases interspecific competition and allows resource sharing between them. This is conventionally called the aggregation model of coexistence[126–130]. Gregarious behavior can occur due to attraction to the food source or attraction between conspecifics. In both, olfactory signals play a major role in communication[113]. These signals are majorly aggregation pheromones that are actively synthesized and released to attract conspecifics[113,131]. Some species are known to de novo synthesize and release aggregation pheromones[25,113], while some produce pheromones by deriving precursors from hostplants[23,24,132]. For instance, mountain pine beetles, *Dendroctonus ponderosae*, produce the pheromone trans-verbenol by hydroxylation of α-pinene, a host monoterpene[23]. In some cases, hostplant odors also play a role, including enhancement of the pheromone's effect[21,22,133] (Fig 3.1).

In the Western Ghats forests, we observed conspecific gregariousness of the *Chiridopsis* spp. on their hostplants. We noted that hostplant individuals with no beetles on a given day were unlikely to have beetles on the following day. On the other hand, whenever hostplants had a few beetles (first visitors), the same plants had a large number of beetles by the next day (Fig. 3.2A, B). Even when heterospecific *Chiridopsis* spp. shared a common host *Ipomoea* sp., a particular hostplant individual always had only beetles of a single species. These observations led us to investigate the presence of an aggregation signal in these beetles that calls conspecifics to the food source. Since aggregation was only observed on hostplants after initiation of herbivory by the first visitors, we asked whether herbivory-induced plant volatiles could function as aggregation signals in these beetles. We proceeded to determine the nature, origin, and composition of this signal.

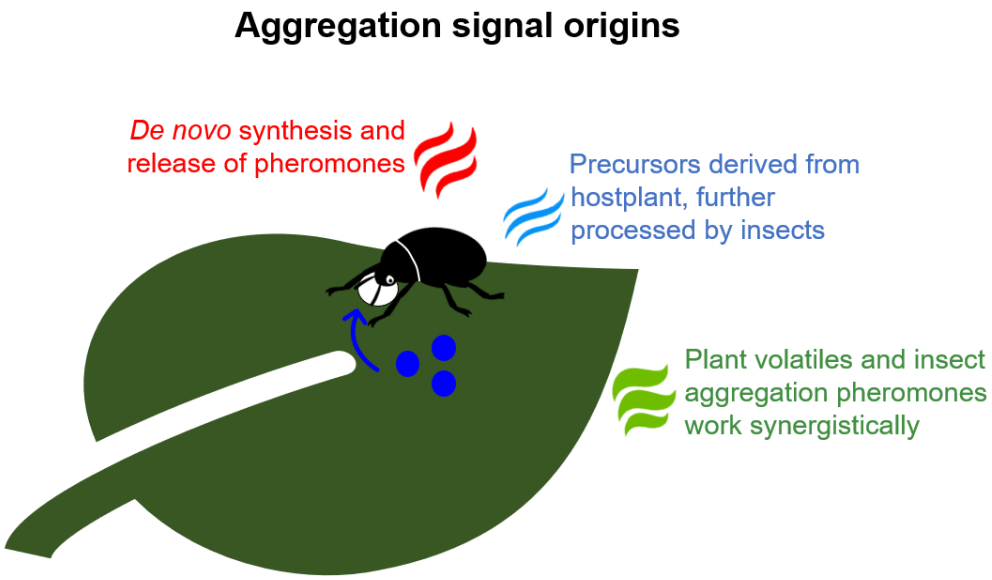

Figure 20: Insect aggregation signals. The chemical signals used by insects for aggregation can have various origins. Some insects *de novo* synthesize and release pheromones to call conspecifics to the food source. Some others derive precursors from the hostplant and chemically modify them to produce the functional signal molecules. In some other cases, host plant odors attract conspecifics, and sometimes synergistically work with pheromones.

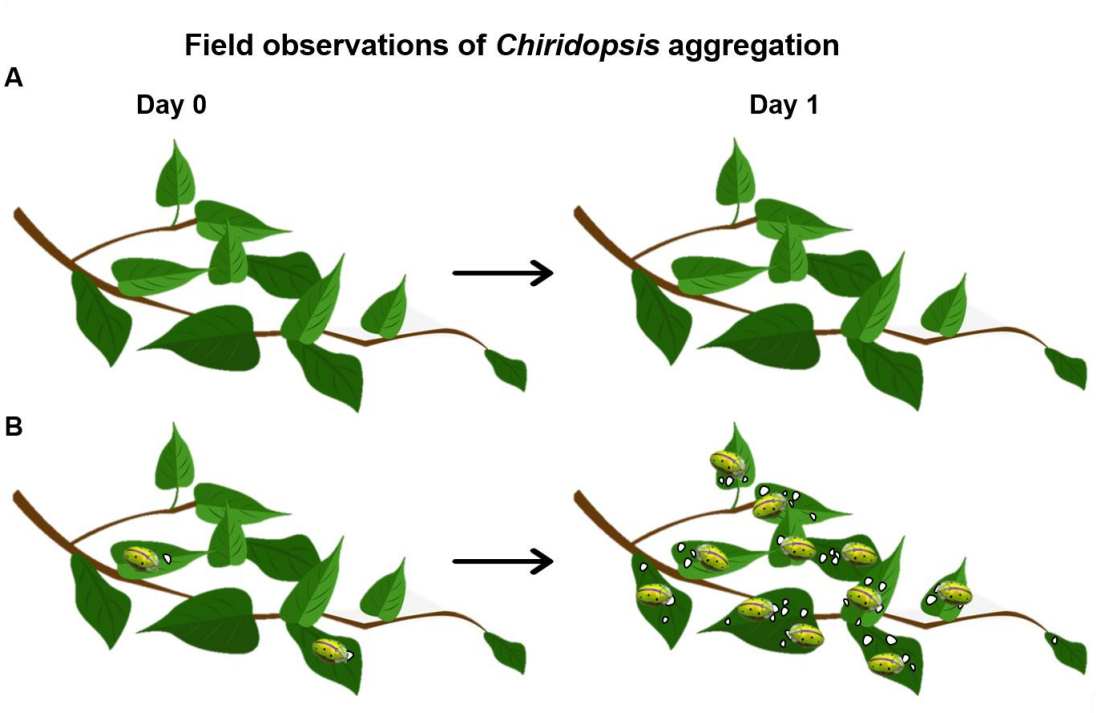

Figure 3.2: Field observations on *Chiridopsis* aggregation. We frequently observed the *Chiridopsis* spp. aggregate in large numbers on their hostplants in the wild. (**A**) Hostplants with no beetles on a given day had no or very few beetles by the next day. (**B**) Contrarily, plants with a few initiator beetles had a large number of beetles the next day.

3.2 Materials and Methods

3.2.1 Plants

Seeds and seedlings of *I. elliptica* were collected from in and around Pune and maintained under controlled conditions in a climate chamber, as described in Section 2.2.1.

3.2.2 Insects

Adults and larvae of *C. nigropunctata* were collected and reared on fresh *I. elliptica* twigs in an insectarium, as described in Section 2.2.2.

3.2.3 Field observations on natural aggregation

To quantify our field observations on *Chiridopsis* aggregation, we counted beetle numbers on hostplants in natural habitats across two days. For each *Chiridopsis* sp., 10 individuals of each hostplant species were surveyed for beetle numbers on a given day (Day 0). The same individuals were revisited 24 h later (Day 1) and surveyed for beetle numbers.

3.2.4 Dual choice assays without visual cues

To understand the nature and origin of the *Chiridopsis* aggregation signal, we conducted experiments using *C. nigropunctata* and its hostplant *I. elliptica* as a model system. To test if visual cues are required for *C. nigropunctata*'s aggregation, we conducted dual choice assays and observed whether aggregation occurred when the hostplants containing initiator beetles were visually occluded. We used plants with untreated (control) and herbivore-wounded leaves. Oral secretions of some other beetles have been found to contain plant defense elicitors which induce the plant's volatile emission[134]. Since the effects of *Chiridopsis* spp. oral secretions are not reported, we also included the mechanically-wounded plant as a control to differentiate between the volatile emissions induced by beetle oral secretions and mechanical damage. Two choices, each placed at the end of one arm of a Y-tube, were provided to five beetles in each assay (I h duration). The choices were visually occluded using barricades made of filter papers Beetle preference was calculated as the number of visitors on a particular choice (n= 5 assays) (mean± SE).

3.2.5 Dual choice assays with and without the initiator individual

To understand whether the aggregation signal consists of VOCs originating from beetles who first colonize the hostplant, we analyzed if beetles preferred to visit hostplants having an initiator beetle. Two hostplants of the same treatment (untreated, mechanically wounded or herbivore-wounded) were provided to the beetles at a time, where the two choices differed only in the presence of an initiator beetle. As an initiator beetle, we placed a *C. nigropunctata* adult on one of the choices. Until the start of the assay the initiator was fed on leaves of the hostplant to allow the biosynthesis of hostplant-derived aggregation signal, if any. In the assay, the beetle was caged inside a mesh and placed on the test leaf to prevent the leaf wounding by it. This ensured that the test beetles would receive olfactory signals together from the test leaf and the initiator beetle. In each such assay, five test beetles were released in the Y-tube. Beetle preference for each choice was considered as the number of settlers on that choice in 1 h assay duration (n= 5 assays) (mean± SE).

3.2.6 Dual choice assays to test individual beetles' preferences

We tested individual beetles' preference of visiting untreated, mechanically wounded, and herbivore-wounded hostplants in Y-tube dual choice assays. Two choices were provided at a time and a single *C. nigropunctata* beetle was released into the Y-tube and allowed to make a choice. Beetle preference for each choice was calculated as the number of visitors on that choice (n= 5 assays, each containing 25 different beetles) (mean± SE).

3.2.7 Dual choice assays to test aggregation preference

We tested *C. nigropunctata*'s aggregation preference between untreated, mechanically wounded and herbivore-wounded hostplants in similar dual choice assays as described in Section 3.2.6. In each assay, five beetles were released into the Y-tube, and we counted the number of beetles on each choice at the end of 1 h. Aggregation preference for each choice was calculated as the number of visitors on that choice (n= 10 assays) (mean± SE).

3.2.8 Profiling *I. elliptica*'s induced odor blend

To find candidate wound-induced aggregation signals from hostplants, we studied the temporal kinetics of VOCs in intact, mechanically-wounded and *C. nigropunctata*

herbivory-wounded *I. elliptica* plants. A single beetle was caged on a leaf of an *I. elliptica* plant, and allowed to feed for 0, 12, and 24 h. An intact and mechanically wounded plant were also used. At each timepoint, the treated leaf was cut from the plant and added to 10 mL DCM to extract VOCs as described in Section 2.2.7. VOC extracts were concentrated and run on a gas chromatograph (7890B GC system, Agilent Technologies, Wilmington, DE, USA) coupled to a mass spectrometer and flame ionization detector (7000D GC/triple quadrupole and FID, Agilent Technologies, Wilmington, DE, USA) as described in Section 2.2.8.

3.2.9 SPME analysis of headspace volatiles

Presence of the candidate aggregation signals [α-copaene (αCOP), β-copaene (βCOP), and δ-cadinene (δCAD)] in the *I. elliptica* headspace was ascertained by SPME. This was done using an SPME fiber assembly to collect headspace volatiles, as described in Section 2.2.11.

3.2.10 Complementation assays using candidate aggregation signals

Of the three candidate aggregation signals αCOP, βCOP, and δCAD, we could procure commercially available pure standards of αCOP and δCAD, and tested them in complementation assays. Both the candidate compounds were tested for aggregation initiation activity by exposing beetles to their wound-induced concentrations detected in the GC-MS-FID analysis (n= 10). For this, the mean of herbivore-induced and mechanical wound-induced concentrations of each compound were considered. Beetles' preference was assayed when provided a choice between solvent-pasted (control) and candidate compound-pasted *I. elliptica* leaves. Similar to as described in Section 2.2.10, these experiments were also performed with artificial leaves and artificial leaves bearing *I. elliptica* odor blend. Each choice was placed in the arm of a Y-tube and ten beetles were released. The beetles were allowed to move through the Y-tube, and at the end of 1 h, we counted the number of beetles on each choice. Aggregation preference was calculated as [(no. of beetles on each choice/ no. of beetles used in the assay) × 100] (n= 10).

3.2.11 Statistical analyses

The homogeneity of quantitative data (number of beetles on different *Ipomoea* spp., choice assays, plant volatile concentrations) was tested using Levene's test. Of these, the normal homogenous data were analyzed by unpaired t-test or one-way ANOVA with Tukey's post hoc test ($p \leq 0.05$). Normal non-homogenous data were analyzed by Welch's t-test ($p \leq 0.05$).

3.3 Results

3.3.1 *Chiridopsis* spp. conspecifics aggregate on hostplants in nature

Observations in natural habitats were that the *Chiridopsis* spp. aggregate on their hostplants. We observed that when a hostplant individual had no beetles on Day 0, the same plant rarely had beetles on Day 1 (See Materials and Methods). Contrarily, hostplants that had 1-5 beetles on Day 0 had a large number of beetles by the next day (Fig. 3.3A-D). Striking observations about this behavior were 1) only conspecifics aggregated, and 2) only on hostplants where the first visitors had already initiated herbivory. We proceeded to investigate the presence of an aggregation signal, its nature (visual or olfactory), and its origin (hostplant, beetle, or both), using *C. nigropunctata* and its hostplant *I. elliptica*.

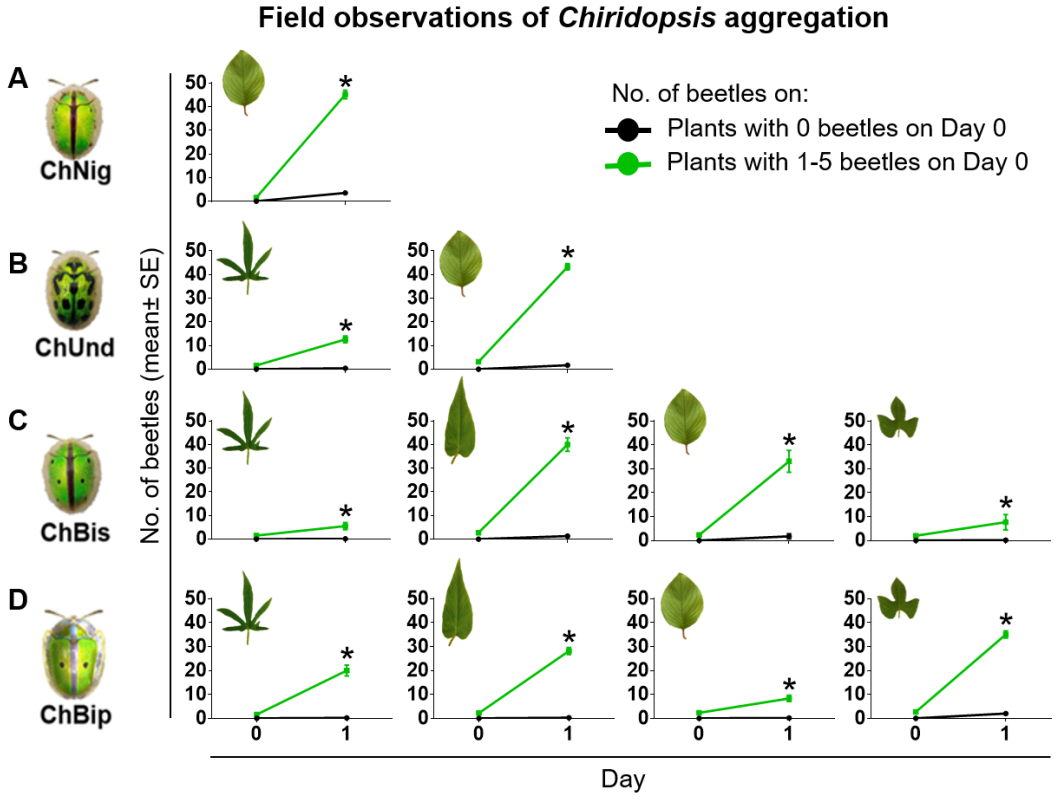

Figure 3.3: *Chiridopsis* spp. are gregarious in nature. As shown in Fig. 3.2, we observed aggregation of *Chiridopsis* spp. on their hostplants in natural habitats. The gregarious behavior was observed for (**A**) *C. nigropunctata*, (**B**), *C. undecimnotata*, (**C**) *C. bistrimaculata* and (**D**) *C. bipunctata*. Striking observations were that the aggregation was always conspecific and only on hostplants where the first visitors had initiated herbivory. Ten plants were surveyed per *Chiridopsis* sp., and data shown is mean number of beetles counted.

3.3.2 The *C. nigropunctata* aggregation signal is not visual

When hostplants with initiator beetles were visually occluded (Fig. 3.4A), we observed that *C. nigropunctata* still aggregated on them (Fig. 3.4B-D). When tested between untreated and wounded hostplants, beetles assembled on mechanically wounded and herbivore-wounded plants (82% ± 9.8 visitors) (Fig. 3.4B, C), but not on intact plants. There was no preference between the two types of wounding (Fig. 3.4D), with beetles visiting both choices similarly. Since aggregation occurred even in the absence of visual cues, these results suggested that the behavior is not initiated by visual signals, and that the signal could be olfactory.

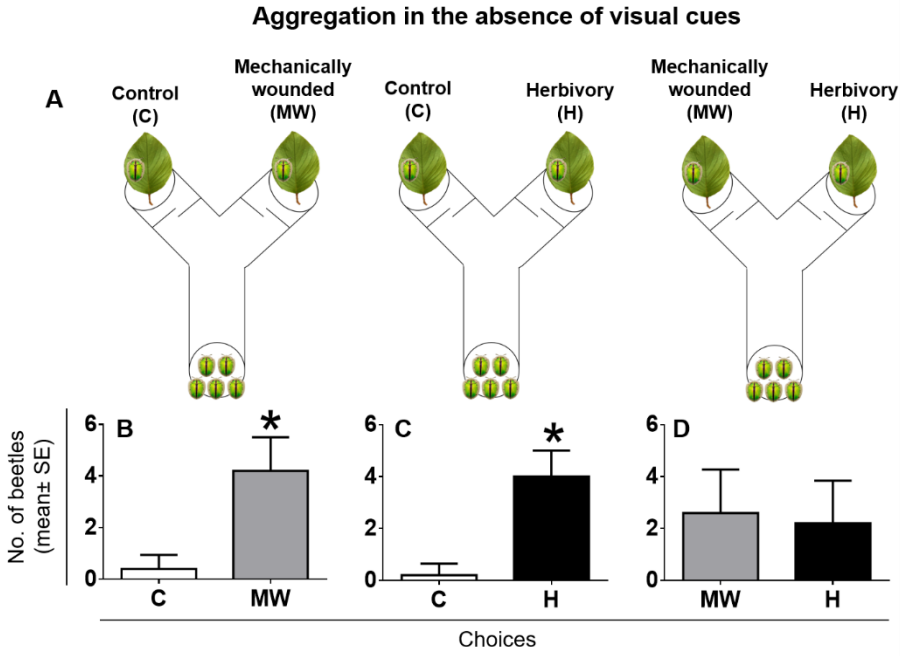

Figure 3.4: The *C. nigropunctata* signal is not visual. *C. nigropunctata* aggregation was tested in the absence of visual cues. (**A**) We observed beetles' aggregation behavior when presented with untreated (control), mechanically wounded, and herbivory-wounded plants, in dual choice assays where each choice was visually occluded using filter paper barricades. In each assay, five beetles were released into a Y-tube and the number of visitors on each choice were counted after 1 h. (**B**) to (**D**) Even in the absence of visual cues, beetles were observed to aggregate, indicating that the aggregation signal is not visual. Aggregation occurred on mechanically wounded (**B**) and herbivory plants (**C**) but not on control plants, with no preference between the two types of wounding (**D**). Asterisks denote significant difference ($p \leq 0.05$ respectively, t-test, n= 5).

3.3.3 *C. nigropunctata*'s aggregation signal originates from a wounded hostplant

To understand whether the aggregation signal originates from initiator beetles who first colonize the hostplant, we conducted choice assays in the presence and absence of the initiator beetle (Fig. 3.5A). Beetles similarly visited the hostplants regardless of the initiator beetle's presence, and this was consistent for untreated (Fig. 3.5B), mechanically wounded (Fig. 3.5C), and herbivore-wounded plants (Fig. 3.5D). Even when there was no hostplant, similar visits were paid to the Y-tube arm with and without a beetle (Fig. 3.5E). Since the initiator's presence did not affect *C. nigropunctata*'s preferences, we inferred that the aggregation is not prompted by a beetle-origin signal. We noted that whenever the hostplant choices were wounded, more beetles paid visits to both choices in the assays (Fig. 3.5C, D), than in other cases where only a few beetles visited either choice (Fig. 3.4B, E).

When similar behavioral assays were performed with individual beetles, we observed the same pattern; beetles showed a significant preference for wounded hostplants over untreated ones, but did not distinguish between the different types of wounding (Fig. 3.6A, C). This trend was also observed while aggregating (Fig. 3.6B, D). Beetles showed equal aggregation on mechanically wounded and herbivore-wounded hostplants but not on untreated ones, with wounded plants receiving ~63% ±7.3 more visitors than intact plants. Since there was no preference between the two kinds of damage, we hypothesized that the *C. nigropunctata* aggregation signal originates from a wounded hostplant.

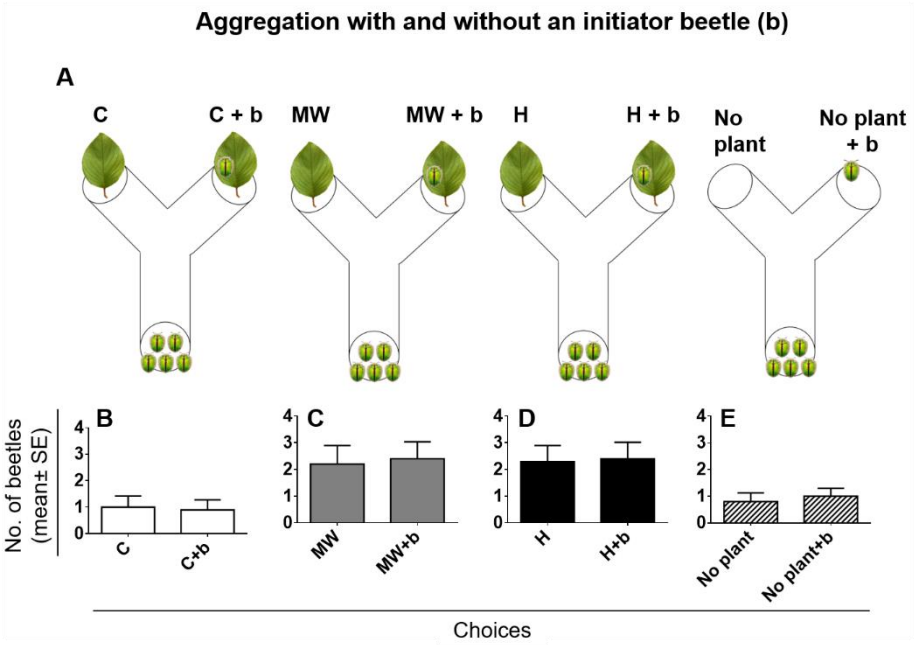

Figure 3.5: The aggregation signal is not of beetle origin. *C. nigropunctata* aggregation preference was tested in the presence and absence of an initiator beetle. (**A**) In dual choice assays using untreated (control), mechanically wounded, and herbivory-wounded plants, we observed beetles' aggregation behavior when the choices differed only in the presence of an initiator beetle. In each assay, five beetles were released into a Y-tube and the number of visitors on each choice were counted after 1 h (n= 5). (**B**) to (**D**) Beetles visited all choices similarly, irrespective of the presence of an initiator. More visits were paid to the two types of wounded plants (**C**) and (**D**) than when the plant was unwounded or absent (**B**) and (**D**). Since there was no preference of aggregation when an initiator beetle was present, we inferred that the aggregation signal is not of beetle origin.

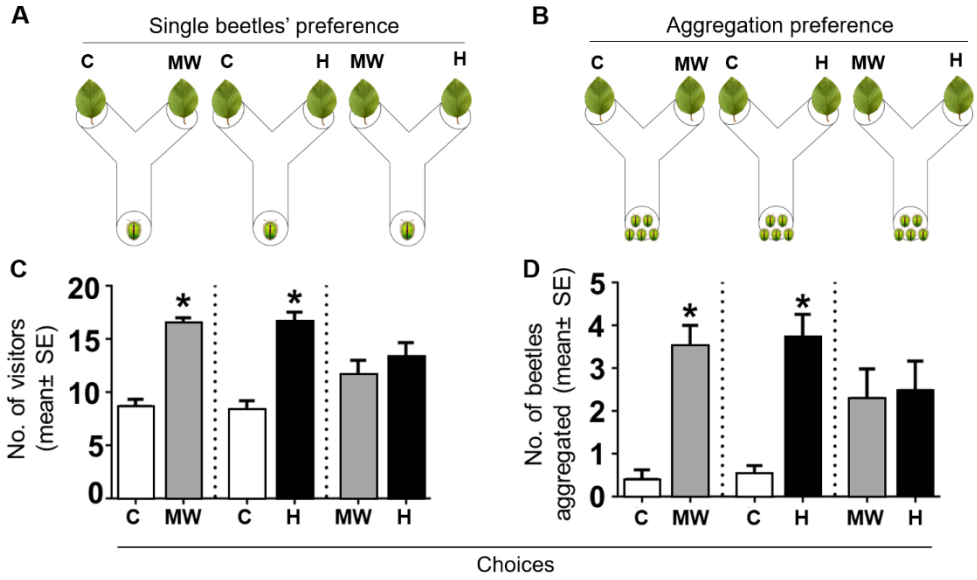

Figure 3.6: The aggregation signal is of wounded hostplant origin. (**A**) to (**B**) *C. nigropunctata*'s preference of visiting and aggregating was tested in dual choice assays. (**C**) In dual choice assays, we observed that individual beetles preferred to visit mechanically wounded and herbivory leaves over untreated ones (n= 5, each with 25 different beetles). (**D**) Beetles aggregated similarly on mechanically and herbivore wounded plants (n= 10), suggesting that the signal originates from a wounded hostplant. Asterisks denote significant differences ($p \leq 0.05$ respectively, t-test).

3.3.4 Three wound-induced sesquiterpenes, α-COP, βCOP, and δCAD, are candidate aggregation signals

To find candidate aggregation signals, we studied the temporal kinetics of *I. elliptica*'s odor blend in response to wounding. VOCs extracted in DCM were analyzed at 0, 12 and 24 h after initiation of herbivory and mechanical damage (n= 6 leaves per treatment). Of all the analyzed compounds, we found that a few sesquiterpenes similarly responded to both wounding types (Fig. 3.7A-E). Of them, only αCOP, βCOP and δCAD were significantly increased in both mechanically damaged and herbivory leaves (2-fold, 2-fold, and 1.89-fold respectively) (Fig 3.7A-C), making them candidate aggregation

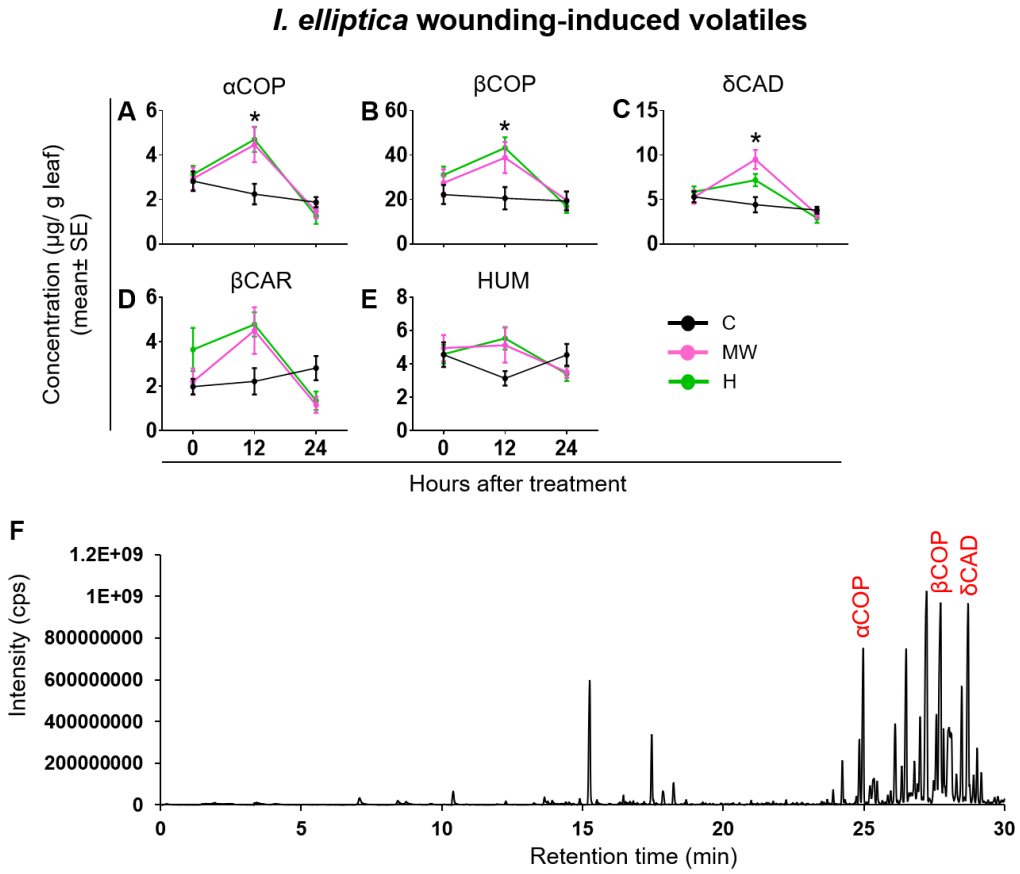

Figure 3.7: αCOP, βCOP, and δCAD are candidate aggregation signals. (**A**) to (**E**) Temporal kinetics of *I. elliptica* sesquiterpenes upon mechanical wounding and herbivory. Three sesquiterpenes, (**A**) αCOP, (**B**) βCOP, and (**C**) δCAD levels were induced similarly in mechanically wounded and herbivore-wounded leaves 12 h after the treatment. As previous experiments suggested that the *C. nigropunctata* aggregation signal is of wounded hostplant origin (Fig. 3.6), these three compounds were candidate aggregation signals. Asterisks denote significant difference ($p \leq 0.05$ respectively, one-way ANOVA, n= 6). (**F**) αCOP, βCOP, and δCAD were also detected in the *I. elliptica* headspace, indicating that they are indeed released into the environment and can function as air-borne communication signals to call conspecifics to the hostplant.

signals. The presence of these three compounds in the *I. elliptica* headspace was also ascertained by SPME (Fig. 3.7F).

3.3.5 αCOP is *C. nigropunctata*'s aggregation signal

Two candidate signals, αCOP and δCAD, were tested in behavioral experiments (Fig. 3.8A-C) to find if they could initiate aggregation on their own or in combination. When *C. nigropunctata* beetles were subjected to dual choice assays between solvent-complemented (control) and compound-complemented artificial leaves (see Section 3.2.10), we observed that they responded to αCOP but not δCAD (Fig. 3.8D, G). Beetles aggregated on αCOP-complemented paper (60%± 7.3 beetles) but not solvent-complemented paper. However, no such preference was shown with δCAD, where beetles similarly visited solvent-complemented and δCAD-complemented choices. Similar results were obtained when the compounds were pasted on *I. elliptica* leaves (Fig. 3.8E, H). Beetles were found to aggregate on *I. elliptica* leaves containing induced levels of αCOP (60%± 3.3 beetles, i.e., ~2-fold higher than control), but not induced levels of δCAD (49%± 5.9 beetles, almost equal to control). The same behavior was observed when the compounds were complemented on artificial leaves bearing the *I. elliptica* odor (Fig. 3.8F, I). When the beetles were presented with the induced concentrations of both αCOP and δCAD together, they showed a similar trend, aggregating on the compound-complemented choices (~2-fold higher than control) (Fig. 3.8J-L). However, these results were comparable to when αCOP was presented alone, suggesting that αCOP and δCAD do not exert a combined effect on aggregation that is more enhanced than individually.

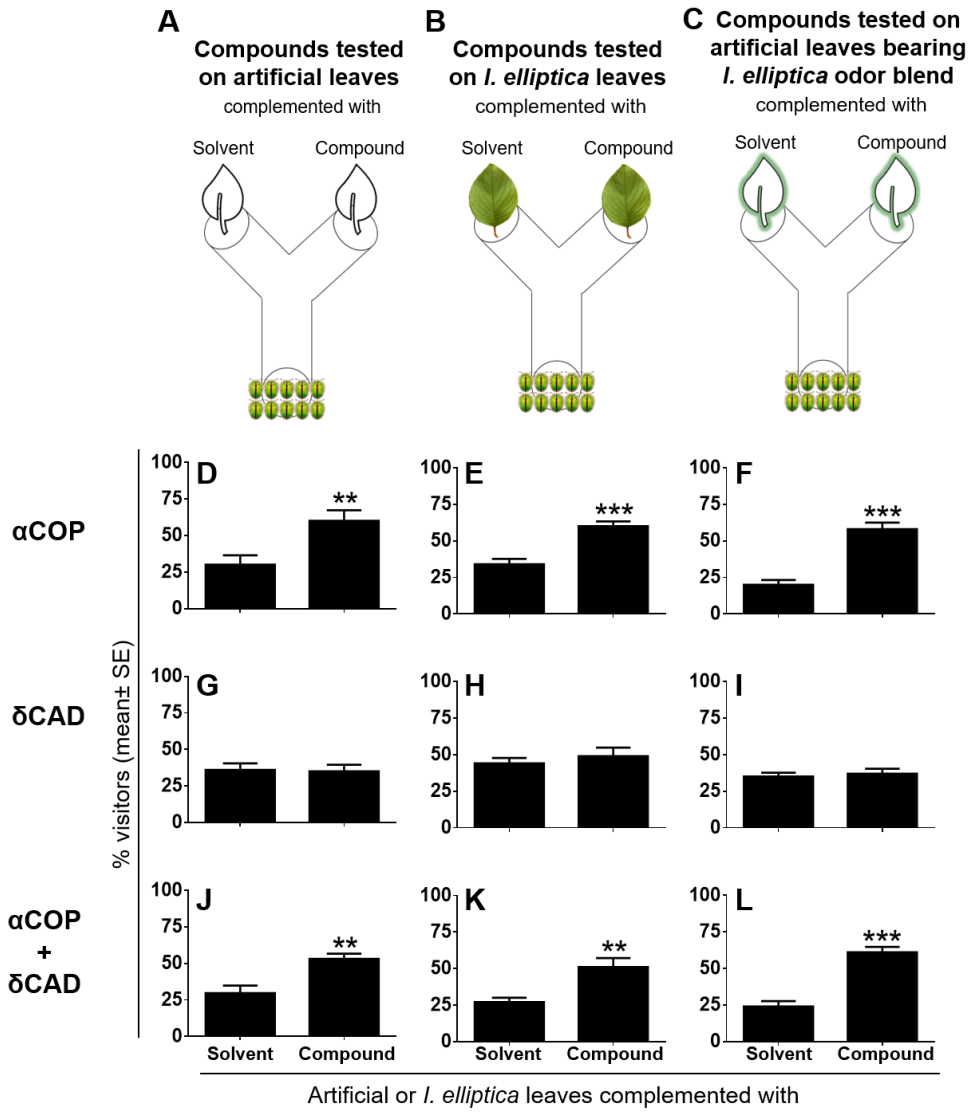

Figure 3.8: αCOP is the aggregation signal. (**A**) to (**C**) Schematic of dual choice assays where candidate signals αCOP and δCAD were tested at their induced concentrations. In each assay. 10 beetles were released into a Y-tube bearing choices of solvent-complemented and compound-complemented leaves. Assays were done using (**A**, **D**, **G**, **J**) artificial leaves, (**B**, **E**, **H**, **K**) *I. elliptica* leaves, and (**C**, **F**, **I**, **L**) artificial leaves bearing *I. elliptica* odor blend. *C. nigropunctata* beetles aggregated on (**D** to **F**) αCOP-pasted choices, even without the background odor of hostplant I. elliptica (**D**, **E**). However, no such response was seen on (**G** to **I**) δCAD-pasted choices, as the beetles equally visited solvent-pasted and δCAD-pasted choices. Beetles also aggregated on choices when αCOP and δCAD were pasted together (**J** to **L**), suggesting that the presence of αCOP is enough to initiate aggregation. Asterisks denote significant differences ($p \leq 0.05$ respectively, t-test, n= 10).

3.4 Discussion

Our observation in the Western Ghats forests was that the *Chiridopsis* spp. aggregate on their hostplants in natural habitats. We noted patterns in their gregarious behavior: only conspecific *Chiridopsis* spp. aggregated on an individual plant, and this occurred only on hostplants. Further, this was only on hostplants where the first few conspecific visitors had initiated herbivory the previous day and not on nearby un-attacked hostplants. These field observations suggested the presence of an aggregation signal to call conspecifics to the food source. We proceeded to investigate this signal's nature, origin, and composition using *C. nigropunctata* and its hostplant *I. elliptica* as a model system. Under controlled conditions, beetles aggregated in the direction of hostplants even when the hostplants were concealed by barricades, suggesting that the aggregation signal is not associated with visual cues and could be olfactory. Since aggregation in natural habitats was only on pre-colonized hostplants, we hypothesized that the first visitors play a role in attracting more beetles to the food source. However, behavioral assays under controlled conditions revealed that beetles showed no preference of moving towards hostplants that already had a beetle, indicating that the initiator insects are not required to initiate aggregation and that the signal is not of beetle-origin. We further found that *C. nigropunctata* beetles preferred visiting and aggregating on wounded hostplants compared to intact ones. That this behavior occurred even in the absence of initiator beetles, without discriminating between mechanically wounded and herbivore-wounded hostplants, suggested that the beetles are attracted to a signal originating from wounded leaves. It also suggested that beetles' frass and oral secretions on the leaves did not affect aggregation. From these experiments, we inferred that a potential aggregation signal must be a compound or group of compounds similarly induced in hostplant leaves by both types of wounding. We proceeded to study the temporal kinetics of *I. elliptica*'s wound-induced odorants. We found three sesquiterpenes to be similarly induced in mechanically wounded and herbivore-wounded leaves, making them candidate aggregation signals. Their putative role as air-borne olfactory signals in initiating aggregation were supported by their presence in the *I. elliptica* headspace.

We tested if αCOP or δCAD could initiate *C. nigropunctata* aggregation on their own or in combination. We found that wound-induced levels of αCOP could initiate beetle aggregation when provided alone, but δCAD could not. Aggregation was similar when

the two compounds were provided together, eliminating the possibility that δCAD is effective only in combination with αCOP, or that they synergistically work. Together, all these results indicate that wound-induced hostplant VOC, αCOP, is the *C. nigropunctata* aggregation signal. Plant VOCs have been shown to play a role in insect aggregation, enhancing pheromone attractiveness[97,133,135,136]. αCOP is a ubiquitous plant VOC, found in a wide range of plants[137]. Several other studies have also reported that this sesquiterpene affects insect behavior by attracting insect herbivores[21,138,139], enhancing mating[21,137,139], and has also been used as lures to trap pests[138,140]. Our study found that the wound-induced level of αCOP is associated with the primary attraction of *C. nigropunctata* to the food source and that this is initiator beetle-independent. It is interesting that this call to conspecific herbivores and subsequent group-feeding on the hostplant is brought about by a hostplant VOC itself. It can be inferred that amidst the evolutionary arms race between plants and insect herbivores for a one-upmanship, these beetles have evolved to recognize αCOP as a sign of a wounded hostplant and the presence of conspecifics. That they respond to the single compound as against the host leaf VOC blend that they use for host location indicates that they have evolved to use αCOP as a post-host location signal, which they perceive in the host blend-independent manner. It will be interesting to see if there are αCOP-deplete populations of this host species in the nature and how *C. nigropunctata* interact with them. Unlike the typical aggregation behaviors where initiator individuals attract conspecifics using signals, here the *C. nigropunctata* beetles assemble at the food source using only a hostplant cue. However, future studies on beetle VOCs will suggest the potential role of beetle-origin signals (if any) in amplifying the aggregation after the primary αCOP signal[111,141,142].

Notably, beetles similarly responded to αCOP when encountered alone or in combination with the *I. elliptica* odor blend. This finding is also congruent with the pheromone system, where single compounds act as pheromones and effect the behavioral changes. This contrasts our findings in Chapter 2, where signal compounds such as attractants and repellents were effective only when accompanied by the background odor of all the hostplant VOCs. This could be because molecules of high ecological importance, such as aggregation signals, dietary nutrient indicators, oviposition site indicators, or sex pheromones tend to be perceived through a distinct and dedicated processing channel as compared to general odors, so as to activate specific behaviors[143,144]. These cues usually bind to the narrowly tuned olfactory receptors and are processed through labeled lines, in

contrast to the combinatorial code processing for more general odors such as host and non-host blends[143,145]. Labeled line processing occurs independent of the background odors and is thought to result in quicker and more sensitive responses to the stimulus odor[143–145].

The role of hostplant VOCs in the conspecific nature of *Chiridopsis* spp. aggregation could be of ecological importance. Particularly, it is interesting to ask whether heterospecific *I. elliptica*-feeding beetles do not gather on the same hostplant individual because each induces and responds to different volatile aggregation signals from the same *Ipomoea* sp. It is also possible that the four *Chiridopsis* spp. have a common aggregation signal but avoid heterospecific aggregation as a strategy of resource sharing while minimizing conflict and competition in this sympatric system[120]. More experiments could enable our evolutionary understanding of this behavior and provide hints as to whether shifts in olfactory perceptions are associated with sympatric speciation in the *Chiridopsis* spp.

Chapter 4

Summary and Future Perspectives

4. Summary and Future Perspectives

In this project, we tried to explore the factors associated with high hostplant specificity in the sympatric *Chiridopsis-Ipomoea* system, particularly in host identification and aggregation. Our field observations were that the natural occurrences of the four *Chiridopsis* spp. show a distinct preference pattern on the five *Ipomoea* spp. These stringent relationships remained unchanged when tested in laboratory conditions; *C. nigropunctata* was monophagous on *I. elliptica* and *C. undecimnotata* was biphagous on *I. elliptica* and *I. batatas*. *C. bistrimaculata* and *C. bipunctata* were oligophagous, and fed on all *Ipomoea* spp. except *I. parasitica*. We wanted to understand how the beetles distinguish between the closely-related hostplants precisely, even when all the species co-occur.

Using the behavioral assays, we inferred that the primary host identification cue is olfactory. GC-MS-FID profiling of the *Ipomoea* spp. revealed that they are associated with a similar set of VOCs, but in unique proportions and combinations. We used multivariate statistics to study the relationship between the five odor blends and the four *Chiridopsis* spp.'s feeding preferences. This analysis allowed us to predict putative attractants and repellents for each beetle, which we tested by exposing beetles to their serial concentrations. Through a series of assays, we discovered that the *Chiridopsis* spp. respond to attractants and repellents only when they are accompanied by hostplant odor blends, and not when separately encountered. This is suggestive of a matrix effect, where attractant and repellent cues are functional only in the background of neutral compounds. The attractants and repellents were also electroantennographically active and present in the *Ipomoea* headspace, indicating that they can be perceived as contactless olfactory cues to locate hosts from a distance.

To visualize this blend-based olfactory perception, we integrated results from behavior, metabolomics, and statistics to develop a novel odor imaging tool that represents odor blends as color variations specific to each insect. Odor images showed that each *Chiridopsis* sp. perceives each *Ipomoea* odor differently. When odors are similar, their resolution is based on the proportion of attractants and repellents. It was interesting to observe that the same host *Ipomoea* sp. is perceived with different odor images by different *Chiridopsis* spp. This suggests that co-occurring insects have evolved to recognize a common hostplant using different cues. Integration of metabolomic,

behavioral, and statistical analyses to image the odor blend is an important finding of this work. In future, the odor imaging can be finetuned by incorporating measures like odor detection thresholds, volatility, and emission rates. Especially with these additions, odor imaging can be used in the real time host location studies. The odor images clearly show the importance of VOC mixtures over separate VOCs; this knowledge can be relevant to agricultural applications as well. For instance, the efficiency of insect lures and traps can be enhanced by using the hostplant volatiles, instead of using a single attractant or repellent volatile. Such insect traps can aid in better crop protection. Results of this study can be useful to understand the olfactory basis of hostplant identification in plant-insect systems where a hostplant is fed upon by different coexisting insect herbivores. For example, the brassicaceous plant Brassica oleracea (cabbage) is fed upon by several insects, such as the specialists, *Plutella xylostella*[146], *Pieris rapae*[147], and the generalists, *Spodoptera litura*[148] and *Spodoptera littoralis*[149]. Odor imaging in this system could give insights into how these co-occurring specialist and generalist herbivores perceive the same hostplant's odor, including whether they differentially respond to the same odorants, plant developmental stages, varieties, etc. Including different varieties or cultivars of cabbage in the odor imaging could give even more insight into how subtle variations in hostplant VOC composition may be perceived by specialist and generalist insects that share the hostplant.

During our fieldwork in the Western Ghats, we also observed the gregarious nature of the *Chiridopsis* spp. Conspecific beetles aggregated on their hostplants where previous visitors had already initiated herbivory. We explored the nature, origin, and composition of an aggregation signal using *C. nigropunctata* and its hostplant *I. elliptica* as a model system. In behavioral experiments, beetles' grouping behavior was unaffected by the absence of visual cues and initiator beetles, suggesting that the primary aggregation signal was olfactory and not of beetle origin. Beetles preferred to visit and aggregate on wounded hostplants compared to unwounded ones, with similar preference for mechanically wounded and herbivore-wounded plants. These results suggested that *C. nigropunctata* is attracted to an aggregation signal that originates from wounded hostplants.

GC-MS-FID profiling of *I. elliptica*'s wound-induced VOCs showed that three sesquiterpenes, α-copaene, β-copaene, and δ-cadinene were induced in both types of wounded leaves after 12 h. We tested α-copaene and δ-cadinene by exposing beetles to

their induced concentrations and found α-copaene to be the aggregation signal. Notably, α-copaene was able to similarly elicit this behavior when provided alone, in combination with δ-cadinene, or in combination with all other hostplant odorants. We could not test β-copaene as its pure compound was not commercially available; therefore, we cannot comment on its role in aggregation. In the future, profiling beetles' emissions after feeding initiation could help understand whether there are beetle-origin signals that also act as aggregation signal(s).

Since the *Chiridopsis* spp. aggregate on all their hostplants upon wounding, it would be interesting to find whether they respond to different signals from their different hostplants. Further, identifying the aggregation signals in the other *I. elliptica*-feeding *Chiridopsis* spp. could provide insights into whether heterospecific beetles avoid grouping because they induce and respond to different aggregation signals from the same hostplant. Such information could shed light on whether the different identification and aggregation signals are associated with resource sharing and conflict avoidance among sympatric beetle species. Whether such shifts in olfactory perceptions are associated with sympatric speciation can also be investigated in the future.

This work underlines the importance of studying wild systems in natural vegetation to understand how insect herbivores resolve cue mixtures of closely-related co-occurring host and non-host plants. Through this research, we attempted to study insect-hostplant specificity beyond the conventional lens of the specialist-generalist paradigm. In contrast to single plant- single insect systems or agricultural pests in monocultures, studying such wild systems allow us to gain insight into the micro-level of host specialization by comparing several specialist herbivores with slightly differing diet breadths. This in turn allowed us to understand the subtle differences in plant chemistry that are associated with hostplant identification in this sympatric system.

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