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Evolution of the venom system in aculeate hymenoptera

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Abstract

Aculeata is an extremely diverse lineage of hymenopteran insects. The name, aculeate, is derived from the Latin word aculeus meaning stinger. This refers to the defining feature of the group, a weaponised ovipositor. This weaponised ovipositor is an aculeus that is able to inject complex cocktails of bioactive molecules into prey or predators. There are over 70, 000 described species and a wide diversity of life history traits, including parasitoids, predators and pollinivores. Aculeates can be further divided into two general categories; solitary and social. In general, the venoms of solitary insects are non-lethal and paralytic allowing for feeding on prey items. Contrastingly, the venom of social insects has lost its paralytic function and is generally used for defence. Research into the venom of aculeates has only been undertaken on a few medically important species, neglecting a large portion of the group.

The primary aim of this thesis was to elucidate differences in the aculeate venom system as a result of social behaviour. This was accomplished by incorporating complementary approaches of scanning electron microscopy, energy dispersion spectroscopy, proteomics, transcriptomics, functional assays, and phylogenetics, providing a broad look into the complexities of the aculeate venom system.

Chapter One reviews the aculeate venom system, tying together what is already known about the venom-delivery apparatus and venom composition across the group. The chapter highlights the lack of information describing venom components from solitary species and the absence of any major comparative analyses. Chapter Two provides the first insight into the morphological adaptations and metal accumulation of the aculeus in Aculeata. Chapter Three presents an in depth comparative description of the venom profiles of various solitary and social species, revealing striking differences the most likely correlate with venom use. The data explored in Chapter Four reveals the phylogenetic and evolutionary histories of the major allergen families found in hymenopteran venoms, demonstrating that most allergens are under the influence of strong negative selection.

Overall this thesis provides a basis upon which to understand these understudied insect venom systems and will have wide-reaching effects pertaining to the evolution and ecology of stinging bees, wasps, and ants.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications included in this thesis

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Lam, T.	Edited paper (10%)
Douglas, J.	Edited paper (5%)
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aculeata, hymenoptera, venom, evolution, aculei, proteomics, transcriptomics, allergen

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List of abbreviations used in the thesis

1D	One-Dimensional
AFU	Average fluorescence unit
Ag5	Antigen 5
AUC	Area under the curve
Bp	Base pairs
BSA	Bovine serum albumin
CNS	Central nervous system
CO_2	Carbon dioxide
CRiSP	Cysteine-rich secretory protein
Cu	Copper
Da	Daltons
df	Degrees of freedom
dN	Non-synonymous
DNA	Deoxyribonucleic acid
dS	Synonymous
EDS	Energy Dispersive Spectroscopy
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
Fe	Iron
FLIPR	Fluorescent Imaging Plate Reader
FUBAR	Fast Unconstrained Bayesian AppRoximation
g	Grams
GABA	Gamma-aminobutyric acid
h	Hours
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
IgE	Immunoglobulin E
K	Potassium
kDa	Kilo Daltons
keV	Kilo electron-volt
LC-MS	Liquid chromatography-mass spectrometry

MCD	Mast cell degranulating peptide
MEME	Mixed effects model of evolution
Min	Minute
mL	Mililiters
mM	Millimolar
MM96L	Malignant melanoma
Mn	Manganese
mRNA	Messenger Ribonucleic acid
MS/MS	Tandem-mass spectrometry
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
MYA	Millon years ago
m/z	Mass number of ion divided by charge
nAChR	Nicotinic acetylcholine receptor
Na	Sodium
NaCl	Sodium Chloride
NFF	Neonatal foreskin fibroblast
nm	Nanometers
P/B	Peak Background
PGLS	Phylogenetic Generalized Least Squares
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
pH	Potential hydrogen
RMPI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Sec	Second
SEM	Scanning Electron Microscopy
t	T statistic
Ti	Titanium
TPM	Transcripts per kilobase million
Tris-HCl	Tris hydrochloride
tRNA	Total RNA
µg	Micrograms

µl	Microliters
V	Version
Zn	Zinc
µM	Micromolar
ω	Omega
ZAF	Z - atomic number, A - absorption of X-rays in the specimen and F - fluorescence caused by other X-rays generated in the specimen

Note on style to the reader

This thesis is presented as a series of manuscripts that have been published, submitted, or are awaiting final comments from co-authors prior to submission. As a result, there may be repetition across the introductions of each chapter and may be certain inconsistencies between chapters.

CHAPTER 1

Aculeata: The stinging tale of venom evolution and variation among wasps, ants and bees

Manuscript prepared for Toxins

Abstract

The aculeate hymenopterans (wasps, ants and bees) are a unique group of venomous animals. The aculeate venom-delivery apparatus is uniquely derived from the female ovipositor and sex accessory glands, reflecting its derivation from the reproductive systems of endoparasitic wasps. Since the adoption of a venomous lifestyle, massive radiation coupled with changes in reproductive strategies, trophic strategies (of both larvae and adults) and social behaviour have profoundly altered aculeate hymenopteran biology, including the composition and bioactivity of their venoms. Extant aculeates produce a wide range of enzymatic and peptide toxins capable of producing paralysis, pain, and death, allowing them to mediate hunting, defence from predators, and parasitism. These include linear peptide toxins, allergens and enzymes such as phospholipase A_2 and hyaluronidase. In this review, we draw together what is known about the evolution of aculeate venoms, integrating the behavioural, morphological, and biochemical aspects.

1.1. Introduction

Hymenopteran infraorder Aculeata (from the Latin *aculeatus*, 'the stingers'; Figure 1.1.) comprises a hyper diverse assemblage of >70,000 species of bees, wasps and ants worldwide [1]. Aculeates are established in virtually every terrestrial ecosystem worldwide, in which they perform diverse roles as parasitoids, predators and pollinators [2].

Unique among venomous animals, the venom apparatus of hymenopterans is derived from the female ovipositor and sexual accessory glands [3]. Clades basal to Aculeata, such non-aculeate Apocrita and parasitic wood wasps (Orrusoidea), use venom to facilitate endoparasitism of their larvae [4]. In Aculeata, the ovipositor has lost its egg-laying function and has been weaponised as a dedicated venom-delivery apparatus, the aculeus. Throughout aculeate radiation, venom use has been retained in most families and adapted to a wide range of trophic strategies and social behaviours. Only in a few groups has venom use been lost, including the stingless bees (Apidae: Meliponini), two subfamilies of ants (Formicidae: Dolichoderinae and Formicinae) [5], and most members of the solitary wasp family Chrysididae [6].

Figure 1.1. Venomous aculeates (A) Solitary velvet ant, *Dasymutilla klugii* (Vespoidea: Mutillidae) Photo © Bob O'Kennon; (B) Social Asian giant hornet *Vespa mandarinia* (Vespoidea: Vespidae) Photo © Thomas Brown; (C) Solitary leaf-cutter bee *Xylocopa californica* (Apoidea: Apidae) Photo © Mike Lewinski; (D) Eusocial giant Amazonian ant *Dinoponera gigantea* (Vespoidea: Formicidae) Photo © Mr. Instr.

While venom use is almost ubiquitous among some other arthropod groups such as arachnids and centipedes, many insects are non-venomous. Aculeate venoms are the most well-known of the insect venoms and cause a remarkable diversity of physiological symptoms including pain, paralysis, tissue damage, and mortality; in addition, aculeate venoms are strongly allergenic and can produce anaphylaxis in some humans [5]. This review focusses on how venom composition and function relates to the overall biology and life history of aculeate species. A particular emphasis is given to reviewing currently available information on the comparative aspects of venom between solitary and social species; and between venoms used for predation, defence, and parasitism (or combinations of these).

1.1.1. Toxin evolution

Venom has evolved multiple times across the tree of life [7]. Independently evolved venoms frequently exhibit convergent molecular evolution, with particular protein families involved in non-venom physiology recruited multiple times as venom toxins. These convergently-used families include proteins such as phospholipase A_2 (PLA_2), cysteine-rich secretory protein

(CRiSP), and hyaluronidase [7]. Peptides are major components of many venoms, especially disulfide-rich families such as inhibitor-cystine-knots and CSαβ-type defensins [8]. The convergent evolution of toxins across the animal kingdom suggests that there are structural and functional constraints on venom toxin evolution.

Venom proteins may either be selectively expressed in the venom gland after duplication of a gene used in normal physiological processes such as signalling or immune processes [9] or expressed in both venom and non-venom tissues from a single gene locus [10]. Often, toxin-encoding genes have been duplicated to produce multi-gene families, and exhibit accelerated evolution [9, 11-17]. The tertiary structural scaffold of toxins is often conserved, with key functional residues on the surface of the molecule modified to confer new functional properties [15, 18]. Rapid evolution of toxin-encoding genes is thought to be due to increased positive selection and neofunctionalisation [18]. However, the preservation of toxin potency has also been known to occur via negative selection, especially among lineages that evolved venom use anciently [19, 20].

Aculeata diverged from other hymenoptera ~190 million years ago [21, 22], however, no direct information is available about the tempo or mode of toxin-encoding genes in hymenoptera. Chapter Four in this thesis provides the first look at the evolutionary and molecular evolution of the major allergens found in hymenopteran venoms. Demonstrating that the majority of these allergens were evolving under the influence of strong negative selection, consistent with other ancient groups of venomous animals [19, 23, 24].

1.2. Venom apparatus

The venom apparatus in aculeates is derived from the ancestral reproductive system [3] (Figure 1.2.) that has been weaponised to form an efficient system for the production, storage and injection of venom. It consists of the sting (or aculeus) and its associated glands. The aculeus is located at the distal base of the abdomen, derived from interlocking gonocoxal appendages [1, 25]. The morphology of the venom apparatus shows family- and species-specific differences, though the general configuration remains the same. *Apis* species have been found to present with backward sloping barbs on the aculeus as do many species in Vespidae. The presence of these barbs has been postulated to relate to increase the occurrence of aculeus autonomy, the technique of self-amputation of the aculeus [26]. The aculeus in solitary bees, wasps and ants were generally found to lack barbs or serrations [27].

The internal parts of the venom apparatus, comprise of the venom reservoir, tubular secretory glands and Dufour's gland. They are formed from infoldings of valves of the ninth segment [28], which are possibly homologous to the sex collaterial glands of non-hymenopteran insects (Figure 1.2.). The main site of toxin production are the two tubular secretory filaments that open into the venom reservoir, often simply termed the venom glands [29]. These tubular glands are composed of columnar secretory cells, each with cuticular end-apparatus, which are type III secretory units [30]. In Apidae, the tubules become fused before connecting to the reservoir, whereas in Vespidae the tubules remain completely separate. In Sphecidae the tubules present only short outgrowths [31], and in some Thynnidae, the tubules are branched as occurs in some non-aculeate Apocrita [3].

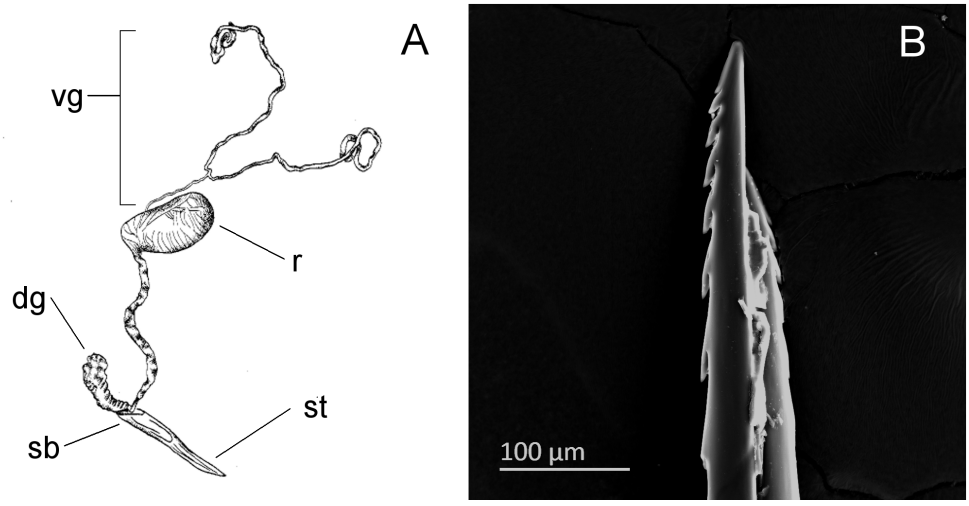

Figure 1.2. Sting apparatus (A) Morphology of the sting apparatus of an ant, *Rhytidoponera* sp., adapted from [3]; vg, venom glands; r, reservoir; dg, Dufour's gland; sb, sting bulb with valves; st, sting. (B) Scanning electron microscope image of *Apis mellifera* sting [27].

The tubular glands empty into the venom reservoir, a bulb-like structure composed of pavementous epithelium internally coated with cuticle [32]. The convoluted gland is a well-developed secretory portion inside the lumen of the venom reservoir and may also contribute to toxin synthesis [32]. The reservoir is connected via the venom bulb containing valve structures to the aculeus.

The Dufour's gland is an exocrine gland composed of type I secretory cells that produces lipids, long-chain hydrocarbons and volatile oxygenated substances. The function of the secretion differs across aculeate families. In ants it is known to be involved in communication and defence [33] while in solitary bees it is implicated in nest building and protection [34, 35]

and in wasps it has been postulated to be involved in kin recognition [36]. In social bees such as *Apis*, it likely serves roles of fertility signalling and nestmate recognition [37]. In ants, the Dufour's gland empties into the venom bulb and hence the aculeus, whereas in other aculeates it empties directly into the dorsal vaginal wall [38].

1.3. Venom function

Aculeates are best known for their use of venom to defend themselves from potential predators. However, venom use for defence primarily occurs in social aculeates, while ancestrally solitary species utilised their venom for reproduction, prey paralysis. Although the adults of most aculeate species subsist on liquid foods such as nectar, the larvae are usually carnivorous endo- or ecto-parasitoids of other arthropods [39-41]. Most of these species retain the ancestral mode of reproduction, in which the female insect injects venom to subdue or paralyse the prey before laying an egg inside or alongside it. However, some groups have shifted to predation or phytophagy in order to provision larvae. Bees (Anthophila) are one such group and provision their larvae with pollen. The role of venom in aculeates is dependent on its life history and trophic strategy leading to an interesting question of whether these differences in functionality translate to differences in composition.

1.3.1. Trophic strategies

Hymenoptera have a large range of trophic strategies that have evolved over millions of years. Most studies on Hymenoptera trophic strategies have been based on the feeding habits of larvae. The most primitive hymenopterans were phytophagous insects; Cynipoidea, or ectoparasites; Orussoidea whose offspring fed on other insects. Apocrita (Symphta and Aculeata) most likely evolved from an ectoparasitic ancestor, with free-living and endoparasitic species arising within the group [42-44]. This suggests that life history strategy shifts have occurred numerous times in hymenoptera with free-living, ectoparasitic, and endoparasitic lifestyles arising on multiple occasions. Aculeata is comprised mainly of solitary parasitoid wasps [45]. The exceptions to this are the groups that have developed social behaviours, Apoidea, Formicidae and Vespidae (Figure 1.3.). Predation in Aculeata has undoubtedly evolved from the ancestral parasitoid mode of life [6, 46]. The shift to predation and pollinivory as trophic strategies coincide with cooperative group living, most likely due to the division of labour within the colony allowing for diversification of feeding behaviours.

1.3.2. Solitary and social aculeate venom

Venoms show numerous adaptations to the specific ways they are used in nature [47]. The original purpose of solitary parasitic hymenopteran venom was a non-lethal paralytic function used in order to paralyse prey or hosts, generally other arthropods, and in defence [4]. Solitary parasitic venom does not have much of an effect on vertebrate predators with the paralytic components found in these venoms targeted to arthropod biology. With the evolution of sociality in aculeates, defence has become a more widespread function and, in some cases, become the primary function, as in the case of many species of Apoidean bees. Social venoms appear to have further evolved to cause pain and augment the immune response in humans and other vertebrate predators [48]. The role of venom in solitary, social, parasitic, parasitoid, predator and pollinivorous species has fundamentally changed over the course of hymenopteran evolution, from acting as a paralytic typically for arthropod predators to the loss of this function and pain becoming the foremost feature. These distinct differences in the function of the venom suggest that the main components of the venom are also likely to be different.

1.3.2.1. Solitary aculeates

Solitary aculeates are ectoparasites that use their venoms to paralyse their prey. The main exception to this is solitary bees, which are pollinivorous and do not use their venom in any predatory capacity. Accordingly, the majority of venom in solitary aculeates is likely to have multiple different functions including paralysis, antimicrobial activity and developmental arrest [49, 50]. There have been some interesting studies done on solitary bee venoms that show that the venoms function as an external immune defence [51]. However, very few studies have been done on solitary species and even fewer components have been characterised [52] due to the lesser medical and economic importance of solitary species relative to social species [49]. Thus, the comparison of toxic component distribution in solitary and social aculeates would provide further insight into the phylogeny and evolution of the group.

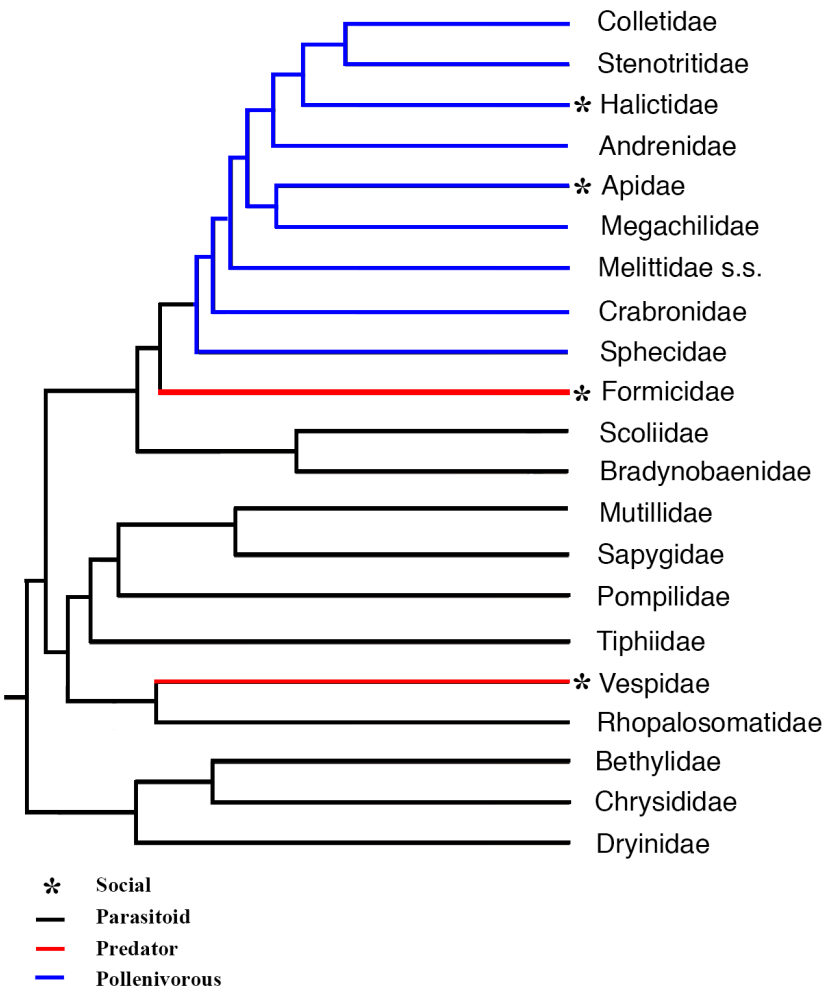

Figure 1.3. Simplified phylogeny of Aculeata based on [53, 54] depicting multiple origins of sociality and life history strategies.

1.3.2.2. Social aculeates

Social aculeates use their venom primarily for defence and in some cases to subdue or kill prey. The effectiveness of a defensive venom depends on the ability to cause immediate pain, tissue damage and potentially death [55]. Pain is the biological sign that damage has occurred and key for the instant defensive efficacy of social aculeate venoms. Due to the varying pain most social aculeate venoms are known to induce it would seem that their venoms have evolved in order to maximise on this pain as their main defensive component [56]. Another effective defence mechanism the social aculeate venom has is its ability to cause anaphylaxis in intruders, notably humans. Various components in the venom possess antigenic properties [57-60]. An anaphylactic reaction in an intruder not only immediately stops the attack but can also infer a learnt response in the intruder to avoid the insect in the future, a valuable lesson for both the insect and the intruder.

1.4. Venom Components

Aculeate venoms are comprised of proteins, peptides and biogenic amines (Tables 1.1. - 1.7.). however, the majority of studies on venom have only examined the venom by isolation of abundant peptides followed by Edman degradation sequencing [61-76]. In contrast the use of transcriptomics and proteomics looking at the venom as whole has only been applied to a few groups [77-98]. From these studies venom peptides such as hyaluronidases, PLA_2, kinins, mastoparan-like and chemotactic-like peptides have been found in both solitary and social aculeate venoms.

1.4.1. Neurotoxins

Neurotoxins have been found in multiple families of aculeates boh solitary and social and are generally utilised in order to rapidly immobilise prey. Neurotoxins in aculeates have been found to act on a wide range of molecular targets, predominately ion channels [99]. Apamin is a neurotoxic compound that has been isolated from the venom of *Apis mellifera*. Apamin is permeable to the blood-brain barrier resulting in direct effects the central nervous system (CNS) [100], as well as being able to selectively block potassium channels [100]. Pompilidotoxins are neurotoxins isolated from the venom of a solitary wasps *Anopolis samariensis* and *Batozonellus maculifrons* [101, 102]. They are both short peptides with no known homology to other toxins that slow fast inactivation of neuronal sodium channels in vertebrates and invertebrates [103]. Philanthotoxins from *Philanthus triangulum* block postsynaptic glutamate receptors and nicotinic acetylcholine receptors [104]. Neurotoxins identified in aculeate venoms can be seen in Table 1.1.

1.4.2. Kinins

Kinins are neurotoxic and pain-producing components found in solitary and social wasp and ant venoms, but not bee venoms. Kinins isolated from the venoms of solitary wasps may play a part in paralysis of prey items. Kinins in social wasps and ants likely play a different role. However, the almost universal presence of kinins in their venoms suggests that kinins function as a defence mechanism by generating pain in vertebrate predators [105]. Reasons for the absence of kinins or kinin-like properties in the venoms of Apidae remains obscure, requiring further characterisation of these aculeate venom proteins in order to answer this question. Kinins identified from the venoms of aculeates can be seen in Table 1.2.

Table 1.1. Neurotoxins of Aculeata

Family	Species	Name	Putative activity	Ref
Solitary				
Crabronidae	*Philanthus triangulum*	Philanthotoxin	Inhibits the release of glutamate and blocks post-synaptic glutamate receptors	[106]
Pompilidae	*Anoplius samariensis, Batozonellus maculifrons*	α-Pompilidiotoxin	Paralysis by Na+ channel blocking	[107, 108]
Pompilidae	*Batozonellus maculifrons*	β-Pompilidiotoxin	Paralysis by Na+ channel blocking	[107]
Vespidae, Pompilidae	*Anoplius samariensis, Eumenes pomiformis, Rhynchium brunneum*	Dendrotoxin-like venom peptide	Paralysis (K+ channel blocking)	[109]
Social				
Apidae	*Apis mellifera, Apis cerana cerana*	Apamin	Blocks voltage-independent calcium-activated K+ channels	[85, 110]
Vespidae	*Vespa orientalis*	Orientotoxin	Neurotoxin presynaptic effect	[111]
Vespidae	*Vespa mandarinia*	Mandaratoxin	Irreversibly blocks the excitatory postsynaptic potential in lobster leg	[112]
Formicidae	*Ectatomma tuberculatum*	Ectatomin	Strong inhibitor of calcium currents in heart muscles	[113, 114]
Formicidae	*Anochetus emarginatus*	U1-poneritoxin	Inhibits calcium channels	[63]
Formicidae	*Paraponera clavata, Anochetus emarginatus*	Poneratoxin	Blocks synaptic transmission in insect CNS, prevents inactivation of voltage gated Na channels	[63, 115]

Table 1.2. Kinins of Aculeata

Family	Species	Name	Putative activity	Ref
Solitary				
Pompilidae	*Cyphononyx fulvognathus,*	Cyphokinin	Inhibits angiotensin-converting enzyme and targets B2 bradykinin receptor, provokes contraction of smooth muscle preparation	[116-118]
Pompilidae, Scoliidae	*Megascolia flavifrons, Colpa interrupta, Megacampsomeris prismatica, Campsomeriella annulata annulata, Cyphononyx fluvognathus and Carinoscolia melanosoma fascinata*	Threonine6 -bradykinin	Inhibits angiotensin-converting enzyme and targets B2 bradykinin receptor, provokes contraction of smooth muscle preparation	[116-118]
Pompilidae	*Cyphononyx fulvognathus*	Fulvonin	Inhibits angiotensin-converting enzyme targets B1 bradykinin receptors and potentiates the smooth muscle contraction elicited by bradykinin	[119]
Social				
Vespidae	*Polistes major major, Parapolybia indica*	Wasp Kinin	Relaxation of smooth muscle	[64]
Vespidae	*Vespa mandarinia, Vespa xanthoptera, Vespula maculifrons*	Vespakinin	Induces smooth muscle contraction	[120-122]
Vespidae	*Polistes lanio*	Tachykinin-like peptide	Unknown	[123]

1.4.3. Cytolytic Peptides

Cytolytic toxins in aculeate venoms are generally short, linear peptides that lack disulfide bonds [124]. They often exhibit antimicrobial and or hemolytic properties [125]. The biological functions of these peptides have not all been elucidated however cytolytic peptides are proposed to cause action potentials in excitable cells quickly leading to cell death [126]. Cytolytic peptides in both solitary and social aculeate most likely act synergistically with other toxins, disrupting cell membranes and rapidly immobilizing prey. Melittin is perhaps the best-known toxic peptide from bee venoms, facilitating ion diffusion across the cell membrane by causing the direct lysis of cells, leading to the sensation of pain [127]. The molecular mechanisms of melittin and other cytolytic peptides remains under debate. However, studies have shown that there is no unique mechanism that melittin performs by, rather the mechanism varies with experimental conditions [128-130]. Cytolytic peptides identified in aculeate venoms can be seen in Table 1.3.

Table 1.3. Cytolytic peptides of Aculeata

Family	Species	Name	Putative activity	Ref
Solitary				
Apidae	*Mellecta albifrons*	Melectin	Antimicrobial, mast cell degranulating and hemolytic activities	[131]
Apidae	*Xylocopa appendiculata*	Xac-1 and Xac-2	Antimicrobial peptide, mast cell degranulating	[132]
Apidae	*Xylocopa appendiculata circumvolans*	Xylopin	Antimicrobial activity	[86]
Halicitidae	*Lasioglossum laticeps*	Lasioglossins	Potent antimicrobial activity, low haemolytic and mast cell degranulation activity, and a potency to kill various cancer cells in vitro	[133]
Halicitidae	*Halictus sexcinctus*	Halictines	Potent antimicrobial activity and low hemolytic activity	[66]
Melittidae	*Macropis fulvipes*	Macropin	Antimicrobial and antifungal activity, moderate hemolytic activity against human red blood cells	[134]
Andrenidae	*Panurgus calcaratus*	Panurgine	Antimicrobial activity	[74]
Colletidae	*Colletes daviesanus*	Codesane	Antimicrobial activity	[131]
Megachilidae	*Osma rufa*	Osmin	Weak cytotoxic, potent antimicrobial activity and mast cell degranulation activity,	[75]
Vespidae	*Orancistrocerus drewseni*	Venom peptide 2	Antimicrobial and hemolytic activity	[135]
Vespidae	*Anterhynchium flavomarginatum micado, Eumenes rubrofemoratus, Eumenes fraterculus*	Eumenine mastoparan-AF	Antimicrobial activity	[136, 137]
Vespidae	*Eumenes pomiformis*	EpVP2a and EpVP1	Antimicrobial activity	[135]
Vespidae	*Oreumenes decoratus*	Decoralin	Antimicrobial activity	[138]
Vespidae	*Orancistrocerus drewseni*	Venom peptide 3	Antimicrobial activity	[139]
Vespidae	*Anterhynchium flavomarginatum micado, Eumenes rubrofemoratus, Eumenes fraterculus*	Eumenitin	Antimicrobial activity	[136, 137, 140]
Pompilidae	*Anoplius samariensis*	Anoplin	Antimicrobial activity	[141]
Scoliidae	*Megascolia flavifrons, Colpa interrupta*	Megascoliakinin	Irreversibly block the synaptic transmission of the nicotinic acetylcholine receptor (nAChR) in the insect central nervous system	[142, 143]
Social				
Apidae, Vespidae	*Apis mellifera, A. dorsata, Apis florea, Vespa velutina nigrithorax, Vespa magnifica, Polistes hebraeus, Vespula maculifrons*	Melittin	Hemolytic, cytotoxic, algogen, cardiotoxic	[144-147]

Vespidae	*Polistes dominulus*	Dominulin	Antimicrobial activity	[148]
Vespidae	*Vespa magnifica*	Chemotactic peptide	Chemotaxis, antimicrobial activity	[149]
Formicidae	*Dinoponera quadriceps, Dinoponera australis*	Dinoponeratoxin	Antibacterial	[61, 82]
Formicidae	*Pachycondyla goeldii, Ectatomma brunneum*	Ponericin	Antimicrobial, insecticidal, and hemolytic activity	[62]
Formicidae	*Myrmecia pilosula, Myrmecia banksi*	Pilosulin	Antimicrobial, cytotoxic, hemolytic activity	[150, 151]
Formicidae	*Tetramorium bicarinatum*	Bicarinalin	Antimicrobial and weak hemolytic activity	[152]

1.4.4. Mast Cell Degranulating Peptides

Mast cell degranulating peptides (MCD), as the name suggests, causes degranulation of the mast cells and is of special interest as they are known to interact with IgE molecules related to allergic reactions [153]. Mastoparan is the only MCD found in solitary venoms, this dichotomy is perhaps due to their increased importance in social venoms as bother potential allergens and pain causing agents. However, the mechanism by which they stimulate mast cell exocytosis remains unclear [154]. MCD have been found to exhibit a wide range of biological activities including strong anti-inflammatory properties at high concentrations, resulting in inflammation and pain [155, 156]. High doses of MCD on the mast cell surface may inhibit histamine release, allowing the MCD to act as an anti-allergic agent [157]. However, at low concentrations it causes mast cell degranulation and histamine release [158]. Mast cell degranulating peptides identified from aculeate venoms can be seen in Table 1.4.

1.4.5. Allergens

Allergens are any material that possess antigenic properties and are able to cause an allergic reaction. The chemical properties leading to allergenicity is not well understood and little is known about the biological activities of most of these proteins. Allergic reactions, which may lead to anaphylaxis, are solely contributed to by social aculeates, in particular *A. mellifera* [57, 159]. Previous research has suggested that the allergenic properties of social aculeate venoms may be of defensive value [160]. Known aculeate allergens are presented in Table 1.5. Many enzymes also present with allergenic activities, Table 1.7.

1.4.6. Other proteins/peptides

Many proteins and peptides found in aculeate venoms, particularly solitary aculeate venoms are not easily categorized as most of them have unknown functionality and biological

activities (Table 1.6.). These proteins and peptides need to be addressed with further research.

Table 1.4. Mast cell degranulating peptides of Aculeata

Family	Species	Name	Putative activity	Ref
Solitary				
Vespidae	*Anterhynchium flavomarginatum micado, Agelaia pallipes pallipes, Orancistrocerus drewseni*	Mastoparan	Hemolytic mast cell degranulator	[139, 161]
Social				
Apidae	*Apis mellifera, A. cerana cerana, Bombus pensylvanicus*	Mast cell degranulating peptide	Degranulation of mast cells	[156, 162]
Apidae	*Bombus lapidaries, B. pensylvanica*	Bombolitin	Degranulation of mast cells	[163, 164]
Vespidae	*Polistes rothneyi, Agelaia pallipes pallipes, Protonectarina sylveirae*	Protonectin	Degranulation of mast cells	[165-167]
Vespidae	*Polybia paulista, Icaria sp., Vespula lewisii, Vespa analis, Vespa mandarinia, Vespa xanthoptera, Vespa tropica, Vespa magnifica, Parapolybia indica*	Chemotactic Peptide	Degranulation of mast cells, chemotaxis of neutropils	[161, 168, 169]
Vespidae	*Polybia paulista*	Venom protein 13	Degranulation of mast cells, hemolytic activity	[161]
Vespidae	*Vespa orientalis*	Venom protein	Degranulation of mast cells	[99]
Vespidae	*Vespa magnifica, Polistes major*	Mastoparan-like peptide	Degranulation of mast cells, antimicrobial activity	[136, 170]
Vespidae	*Polybia paulista*	Polybine	Degranulation of mast cells	[171]
Vespidae	*Vespa crabro*	Crabrolin	Degranulation of mast cells	[172]
Vespidae	*Vespa orientalis*	Histamine-releasing peptide	Degranulation of mast cells	[173]
Vespidae	*Protonectarina sylveirae*	Sylverin	Degranulation of mast cells	[165]
Vespidae	*Protopolybia exigua*	Protopolybiakinin	Degranulation of mast cells	[174]
Vespidae	*Polybia paulista, Protopolybia exigua, Agelaia pallipes pallipes, Polistes rothneyi, Polistes jokahamae, Vespa xanthoptera, Vespa mandarinia, Vespa crabro, Vespa basalis, Vespa tropica, Vespa analis, Vespa orientalis, Vespula lewisi, Vespula vulgaris, Protonectarina sylveirae, Parapolybia indica*	Mastoparan	Degranulation of mast cells	[65, 137, 165-167, 172, 175-177]

Table 1.5. Allergens of Aculeata

Family	Species	Name	Putative activity	Ref
Social				
Apidae	*Apis mellifera*	Icarapin	Allergen	[178, 179]
Apidae	*Apis mellifera*	Api m 6	Allergen	[180]
Formicidae	*Solenopsis invicta*	Cysteine-rich venom protein	Allergen	[181]
Formicidae	*Solenopsis invicta, S. geminata*	Venom allergen 4	Allergen	[181]
Formicidae	*Solenopsis invicta, S.richteri, S.saevissima*	Venom allergen 2	Allergen	[181, 182]
Formicidae	*Solenopsis richteri*	Venom allergen 3	Allergen, antimicrobial activity	[182]
Vespidae, Formicidae	*Agelaia pallipes, Polistes gallicus, Polistes annularis, Polistes exclamans, Polistes gallicus, Polistes annularis, Polistes dominul, Polistes fuscatus, Solenopsis invicta, Pachycondyla chinensis, Vespa crabo, Vespa magnifica, Vespa velutina, Vespa mandarinia, Dolichovespula maculata, Dolichovespula arenaria, Vespa vulgaris, Vespula maculifrons, Vespula flavopilosa, Polybia scutellaris rioplatensis, Polybia paulista, Vespula germanica, Vespula pensylvanica, Vespulavidua, Vespula maculifrons, Vespula squamosa, Pachycondyla chinensis*	Antigen 5	Allergen	[81, 99, 182-189]

Table 1.6. Other peptides of Aculeata

Family	Species	Name	Putative activity	Ref
Solitary				
Apidae	*Xylocopa appendiculata circumvolans*	Xylopinin	Unknown	[86]
Vespidae	*Orancistrocerus drewseni*	Orancis-Protonectin	Potent hemolytic activity	[190]
Pompilidae	*Cyphononyx fulvognathus*	Cd-146	Unknown	[119]
Pompilidae	*Cyphononyx dorsalis*	Cd-125	Unknown	[141]
Pompilidae	*Anoplius samariensis*	As-126	Unknown	[191]
Pompilidae	*Batozonellus maculifrons*	Bm-10	Unknown	[191]
Sphecidae	*Sphex argentatus argentatus, Isodontia harmandi*	Sa-112	Inhibits contraction of the locust oviduct	[192]
Sphecidae	*Sphex argentatus argentatus, Isodontia harmandi*	Sa-12	Unknown	[192]
Ampulicidae	*Ampulex compressa*	GABA	Inhibitory neurotransmitter	[193]
Ampulicidae	*Ampulex compressa*	Alanine	GABA receptor agonist	[193]
Ampulicidae	*Ampulex compressa*	Taurine	Impairs the re-update of GABA from the synaptic cleft	[193]
Social				
Apidae	*Apis mellifera*	Tertiapin	Potently blocks certain types of recombinant inwardly rectifying K^+ channels	[194]
Apidae	*Apis mellifera*	PVF	Growth factor activity	[179]
Vespidae	*Polybia paulista*	Paulistine	Hyperalgesia	[195]
Vespidae	*Vespa magnifica*	Vespin	Contractile activity on iuleum smooth muscle	[144]
Vespidae	*Vespa bicolor*	Kunitz-type serine protease inhibitor bicolin	Inhibits trypsin and thrombin	[196]

Table 1.7. Enzymes of Aculeata

Family	Species	Name	Putative activity	Ref
Solitary				
Apidae, Vespidae	*Xylocopa appendiculata circumvolans, Xylocopa appendiculata, Dufourea novaeangliae, Orancistrocerus drewseni*	Phospholipase A_2	Hydrolysis of lecitins	[86, 132, 197]
Apidae, Halicitidae, Vespidae	*Habropoda laboriosa, Dufourea novaeangliae, Melipona quadrifasciata, Polybia paulista, Vespula maculifrons*	Hyaluronidase	Dissemination	[188, 198-200]
Apidae, Vespidae	*Dufourea novaeangliae*	Serine protease	Allergen, hemostasis, spreading factor	[201-203]
Vespidae	*Orancistrocerus drewseni*	Zinc-metallopeptidase	Hemolytic acitivity	[204]
Crabronidae	*Philanthus triangulum*	Philanthotoxin	Inhibits the release of glutamate and block the post-synaptic glutamate receptors	[106]
Pompilidae	*Anoplius samariensis, Batozonellus maculifrons*	α-Pompilidiotoxin	Paralysis by Na+ channel blocking	[107, 108]
Pompilidae	*Batozonellus maculifrons*	β-Pompilidiotoxin	Paralysis by Na+ channel blocking	[107]
Vespidae, Pompilidae	*Anoplius samariensis, Eumenes pomiformis, Rhynchium brunneum*	Dendrotoxin-like venom peptide	Paralysis by K+ channel blocking	[109]
Social				
Apidae	*Apis mellifera, Habropoda laboriosa*	Carboxylesterase	Lipid metabolism, allergen	[205]
Apidae	*Apis mellifera*	Serine carboxypeptidase	Hydrolyse	[85]
Apidae	*Bombus terrestris, Bombus ignitus*	Kunitz-type serine protease inhibitor	Inhibits plasmin	[206, 207]
Apidae	*Apis mellifera, Apis cerana, Bombus terrestris, Bombus ignitus*	Superoxide dismutase	Destroys radicals	[85]
Apidae	*Bombus ignitus*	Serine protease inhibitor	Peptidase activity	[208]
Apidae, Vespidae	*Apis cerana cerana, Bombus pensylvanicus, Bombus terrestris, Polistes gallicus*	Protease	Serine type peptidase activity	[162, 188, 209]
Apidae	*Apis mellifera, Vespula vulgaris*	Dipeptidyl peptidase IV	Allergen	[57, 205]
Apidae	*Apis mellifera, Bombus terrestris, Bombus pensylvanicus, Apis dorsata, Apis cerana cerana, Bombus ignitus, Bombus hypocrita*	Phospholipase A_2	Hydolysis of lecitins	[162, 209-211]
Vespidae, Formicidae	*Vespula vulgaris, Vespula germanica, Vespula*	Phospholipase A_1	Hydrolysis of phospholipids	[177, 198,

	maulifrons, Vespa basalis, Vespa affinis, Polybia paulista, Solenopsis invicta			212-214]
Apidae, Vespidae	*Apis mellifera, Apis cerana, Vespa crabro, Vespa analis*	Acid phosphatase	Hydrolysis of phosphomonoesters at acidic pHs	[99, 215]
Apidae, Vespidae	*Apis mellifera, Polistes gallicus, Polistes annularis, Vespula vulgaris, Dolichovespula maculata, Apis cerana cerana, Polybia paulista, Vespula maculifrons*	Hyaluronidase	Dissemination	[188, 198-200]
Apidae, Vespidae	*Bombus ignitus, Apis mellifera, Agelaia pallipes, Bombus hypocrita sapporensi, Bombus terrestris, Bombus ardens ardens, Polistes dominula, Tetramorium bicarinatum*	Serine protease	Allergen, hemostasis, spreading factor	[79, 201-203]

1.4.7. Enzymes

Enzymes generally represent the high molecular weight fraction of aculeate venom from 15-50 kDa [216]. They are involved in many levels of venom action by, serving as spreading factors and potentiating the toxic action of other molecules. Enzymes are also known to frequently induce allergic reactions [217-222]. Phospholipases are a major enzyme found in aculeate venoms [223, 224]. Phospholipase A_2 (PLA_2) is able to induce the release of IgE-independent mediators [225]. It has also been reported that PLA_2 enzymes can affect a range of cells related to nociception [226] and can contribute to delayed *in vitro* and *in vivo* neurotoxic effects [227]. Acid phosphatase is another enzyme common in aculeate venoms and causes histamine release from sensitized human basophils, as well as an acute swelling and flare reaction [57, 228-230]. The enzyme hyaluronidase is common in both solitary and social venoms due to its role in venom spreading. This is achieved by the degradation of hyaluronic acid in the extracellular matrix, which facilitates the diffusion of the venom [231]. Fragments of hydrolysed hyaluron stimulate inflammation, angiogenesis, and immune response resulting in a quicker systemic envenomation [232]. Known aculeate enzymes are presented in Table 1.7.

1.4.8. Non-proteinaceous components

Aculeate venoms also contain a number of pharmacologically active components, which are responsible for immediate pain; histamine, serotonin, acetylcholine and catecholamines. Histamine is a major component in all aculeate venoms and is important mediator of hyper-sensitive reactions [233], pain and the facilitation of the spread of venom via increased

vascular permeability . The administration of anti-histamines has been shown to reduce pain in insect stings, suggesting that it is one of the pain-inducing components in aculeate venoms [234]. Similarly, catecholamines; dopamine and noradrenaline increase heartbeat, resulting in increased venom circulation and distribution. Serotonin is an irritant and likely contributes to the pain caused by the venom [235]. Acetylcholine stimulates pain receptors synergistically with histamine, which can result in increased perceived levels of pain [158, 236]. Formicidae venoms also contain alkaloids, which make up 80-90% dry weight of venom [237, 238]. These venom alkaloids play a main role in their ecology by aiding manipulation of host species and competitors as well as being able to cause local haemolytic and necrotic effects and pain [59, 124].

1.5. Evolution of venom toxins in aculeates

The venoms of aculeates have a diverse range of functions with many different selection pressures acting on venom toxins. Ichneumonidea are a group of parasitoid wasp species basal to aculeates, and comprised of ecto- and endoparasitic species [21]. The venoms of these parasitoid wasps are primarily targeted towards ensuring successful parasitism and facilitating successful development of their offspring. They use the viruses to ensure the survival of their offspring, through suppression of the immune system, modifications in host physiology, and the eventual death of the host [239, 240]. In contrast aculeate venom is no longer used to manipulate the immune system and host physiology, rather it has evolved to be used for predation and defence. Parasitoid and predatory aculeates have venoms that are attuned to the way they hunt and feed; predatory venoms generally cause pain, or a quick death and parasitoid venoms cause paralysis. These lineages within aculeate hymenoptera have accumulated different evolutionary innovations and most likely a result of different toxic components in their venoms. This provides researchers with an ideal model in which to study how venom proteins change depending on social behaviour and trophic lifestyle.

Most solitary wasps are parasitoids, with venoms causing long term, non-lethal paralysis of prey [241]. The venom composition in parasitoid wasps has been shown to have great variation between species. This is likely due to the huge diversity of prey items between species. Necessitating venom proteins specialised to their intended victims. this highlights the importance of further research into this area [242]. The high variability of venom composition reflects the important functional diversification of venom proteins during evolution. Generally the venoms of parasitic aculeates have been found to comprise

neurotoxic peptides, enzymes; phospholipases, hyaluronidase and acid phosphatase, antimicrobial peptides, and a diverse array of low and high molecular weight peptides that most likely contribute to the paralytic effect of these venoms [243]. Various small peptides that have been found in solitary wasp venoms, such as mastoparans and kinins, have neurotoxic, hemolytic, myotoxic and edematogenic acitivities [117, 176, 244]. These peptides have also been identified in social aculeate venoms. This is probably due to their usefulness in general damage of the victim. The vast majority of proteins and peptides are yet to be identified in solitary aculeate venoms and those that have been identified have no known function with no similarity to known proteins.

In contrast to solitary aculeates the venoms of social Apidae and Vespidae is more homogenous at the genus level [57]. Formicidae venoms show slightly more variation, most likely due to the variety of biological roles (e.g. chemical communication agents) that their venoms play in addition to defence and prey capture [5, 245]. While various studies that have focused on determining the venom profile of the more famous aculeates, the majority have been mostly neglected highlighting the need for further research in this area.

Many solitary wasps including Crabronidae, Sphecidae and Pompilidae, will attack, sting and paralyse prey items then lay eggs directly on the bodies, in contrast social wasps, as found in Vespidae, will usually butcher their prey and feed to their larvae. As a result, social wasps are characterised as predators and not parasitoids and do not need their venom to paralyse and preserve their prey. Their venom appears is used very differently and to have evolved in order to maximise pain and augment allergenic responses, similar to bee and ant venoms. However, despite this there are species of solitary wasps that are characterised with painful stings. Peptides and biogenic amines are mostly responsible for local edema, erythema and pain [246]. In contrast, venoms of parasitoid wasps have low concentrations of such peptides [99], which may underlie their lesser pain-inducing qualities [247]. Venom in Formicidae is used primarily for defence. However, as in Vespidae some ant species further use their venom for prey capture [245], in contrast social bees use their venom only in defence, while solitary bees, are pollinivorous and rarely sting. It has been hypothesised that solitary bee venom secretions are used in order to protect the nest from bacterial or fungal infections rather than offense [75].

There is varying knowledge surrounding the morphological and behavioural adaptations accompanying prey specialisation in many aculeates, and almost nothing is known about the corresponding changes in venom composition.

1.6. Future Directions

This review highlights many gaps in our knowledge of aculeate venoms. This is mostly due to the low yield of venom and difficulty in obtaining significant quantities for characterisation. However, recent advances in the development of deep-sequencing approaches, coupled with transcriptomic, proteomic and functional assays based on mass spectrometry and *de novo* sequencing now allows broader investigations using smaller quantities of venom. Targets that are known to be present within predator/prey should be primary candidates when further exploring the functional diversity of toxins in aculeates. The limited number of studies on the venomes and proteomes of aculeates indicates that there are many potential novel bioactive toxins yet to be discovered and explored, that may additionally be used as potential tools for biopesticide and drug development.

CHAPTER 2

Harden Up: Metal acquisition in the weaponized ovipositors of aculeate hymenoptera

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Abstract

The use of metal ions to harden the tips and edges of ovipositors is known to occur in many hymenopteran species. However, species using the ovipositor for delivery of venom, which occurs in the aculeate hymenoptera (stinging wasps, ants, and bees) remains uninvestigated. In this study, scanning electron microscopy coupled with energy dispersive X-ray analysis was used to investigate the morphology and metal compositional differences among aculeate aculei. We show that aculeate aculei have a wide diversity of morphological adaptations relating to their lifestyle. We also demonstrate that metals are present in the aculei of all families of aculeate studied. The presence of metals is non-uniform and concentrated in the distal region of the stinger, especially along the longitudinal edges. This study is the first comparative investigation to document metal accumulation in aculeate aculei.

2.1. Introduction

Aculeata (ants, bees, and stinging wasps) are the most conspicuous of the hymenopteran insects, and are known predominantly for the capacity to inflict a painful sting [248]. Different groups inflict varying amount of pain, with the most severe responses (as perceived by humans) delivered by taxa including bullet ants (*Paraponera*), tarantula hawk wasps (*Pepsis*), and armadillo wasps (*Synoeca*) [249]. Uniquely among venomous animals, the venom apparatus of aculeates is evolutionary derived from the female's ovipositor [3]. The weaponisation of the ovipositor is associated with the evolution of stinging aculeates diverging from parasitic wasps and may have helped drive the enormous radiation of aculeates [250].

The ovipositors of parasitic hymenopterans are subject to considerable abrasion and need to be both hard and wear-resistant, especially in groups that must repeatedly drill through wood or other dense substrates concealing their hosts. Many parasitoids can also flex their ovipositors as they navigate this structure through the substrate to locate their hidden hosts [251]. Research has shown that parasitic ovipositors are enriched with transition metals such as zinc and manganese, which are hypothesised to affect the mechanical properties of the cuticle [252, 253]. In contrast, aculeate aculei are not required to drill through a hard substrate and thus do not have these same requirements for abrasion and wear-resistance. This dichotomy between the use of the ovipositor in parasitic hymenopterans and the aculeus in aculeates suggests that a metal compositional difference may not be unexpected.

The aculeate aculeus is primarily used for the injection of venom. Aculeates can be divided into social and solitary groups and the functions of venoms in each group are different. Solitary aculeates envenomate their prey to paralyse and preserve it, whereas social aculeates often use the aculeus to defend their colonies from vertebrate predators [254, 255], while at the same time retaining some offensive capabilities [256]. Although the importance of the aculeus in driving social evolution within aculeates has been questioned by some (e.g., Kukuk et al. [257]), this difference in function between solitary and social groups does suggest that the aculeus morphology and metal content may also differ in these taxa in a phylogenetic context. Hence, documenting these patterns using comparative methods can provide the phylogenetic context for assessing the role of the aculeus in insect social evolution.

The primary aim of our study was to investigate the morphological adaptations of aculeate aculei and secondly to determine if there are any metals present in the aculei of aculeates. Here, using a comparative approach, we used a scanning electron microscope (SEM) with an energy dispersive X-ray (EDS) detector to characterize the morphology and composition of the aculeate aculeus for the first time. This research is the first step to understanding the evolution of these understudied insect venom systems and how they impact the evolution and ecology of aculeates.

2.2. Materials and methods

2.2.1. Sample Preparation

One specimen of each species in Table 2.1. was investigated. In species with negative findings regarding metal composition, further specimens were analysed in order to provide confirmation. The studied species were selected to provide detailed sampling within several major groups of Aculeata including ants (Formicidae), apid bees (Apidae), vespid wasps (Vespidae), and several other aculeate lineages. Species were selected to match those used in ongoing study on venom composition within aculeates in order to allow comparisons between ovipositor size and venom profiles. All specimens are vouchered at the Smithsonian National Museum of Museum of Natural History, Washington DC; voucher codes are provided in Table 2.1. The aculei were excised from the abdomen and washed in 80% ethanol. Samples were allowed to air dry and then adhered on a conductive double sided (adhesive) carbon tape and carbon coated.

2.2.2. Energy Dispersive X-ray analysis (EDS)

Scanning electron microscopy was performed using the Hitachi S3700-N scanning electron microscope (SEM) in high vacuum mode. Energy dispersive X-ray spectrometry (EDS) was performed using a Bruker XFlash 4010 silicon drift detector using Esprit v1.9.4 software by Bruker. Samples were imaged and analysed using a beam energy of 20 keV at approximately 10 mm working distance. Areas of interest were first imaged, and then regions of interest were selected for EDS analysis. No suitable metal-bearing carbon-rich standard is currently available to match the material being assessed, hence, an interactive standardless peak-background (P/B) ZAF matrix correction was used to estimate compositions within the Esprit software. The P/B ZAF correction routine is well suited to this study given its ability to measure the composition of non-flat specimen geometry.

Hyperspectral X-ray mapping was applied in order to investigate the distribution of the transition metals present. Acquisition conditions for elemental imaging were 15 keV, 10 mm working distance, and 25º tilt towards the EDS detector to increase the take-off angle from 35 to 60º. Specimens tilted in this fashion suffer a reduced nitrogen absorption by carbon, for example, owing to a shortened path length within the aculeus. Specimen tilting toward the detector additionally reduces shadowing effects from the three dimensional aculeus [258]. X-ray images extracted from the hyperspectral data sets are presented as net count maps, with background counts and adjacent peak contributions removed.

2.2.3. Phylogenetic Comparative Analyses

A phylogeny was assembled using inferred evolutionary relationships within various taxa from previous studies [250, 259-272] and was used for all further analyses conducted in R v3.2.5 using the ape package [273] for general handling of phylogenetic and trait data. Ancestral states were estimated and reconstructed over the tree in order to investigate the evolutionary history of the traits and consequently their relation to one another over time. To provide a comparative estimate of the barbs present on the aculeus, the ratio of the height of the barb relative to the base of the barb was calculated. This calculation corrected for any size bias between species giving an independent measure degree that the aculeus was barbed. The degree the aculeus was barbed and metal concentrations (Zn, Fe, Mn, Cu, and Ti) were reconstructed by maximum likelihood in the contMap function in phytools [274]. We then fit PGLS models [275] in caper [276] to test for relationships between metal content and degree of barbs.

2.3. Results

2.3.1. Morphology

Scanning electron micrographs showed striking differences in the morphology of Apoidea and Vespoidea aculei (Figure 2.1. – 2.5.). The tip of the aculeus of *Apis* species is armed with backward sloping barbs (Figure 2.1.). *Bombus* aculei have fewer barbs at the tip and the barbs are much smaller (Figure 2.2.). Barbs on the aculeus shaft of *Xylocopa* species are undeveloped or absent (Figure 2.3.). *Centris* and *Diadasia* bees have a short bump-like protrusion on the end of the aculeus (Figure 2.3.). Barbs on the aculei of Vespidae and Formicidae show a greater degree of morphological variation than found in Apoidea (Figure 2.4. – 2.6.). Ancestral reconstruction showed that barbs present on the aculeus either had a single common ancestor and has been lost in certain groups or has evolved convergently on multiple, perhaps as many as 7, occasions (Figure 2.9.).

2.3.2. Elemental composition and distribution

Iron (Fe), zinc (Zn), manganese (Mn), titanium (Ti) and copper (Cu) were found to be present in the aculei and almost exclusively in the distal region. The metals were found in minor concentrations between 0.02 – 1.5 mass percent (Table 2.1.). Metals were detected in all aculeate families studied (Figures 2.7. – 2.10.). Zinc was detected in the families Formicidae, Mutillidae and Vespidae. Among all metals for which high concentrations were detected, zinc content showed the highest taxonomic diversity, suggesting multiple convergent increased accumulations of this metal content. Copper was identified primarily in *Polistes* species, particularly *Polistes dorsalis*, but also in *Apis mellifera*. Manganese was found in most families with the major exception of Formicidae, but concentrations above 0.3% were only in two species: *Bombus sonorous* and *Paraponera clavata*. High concentrations of iron were found only in the aculeus of the giant vespid species *Vespa mandarinia* while a high concentration of titanium was restricted to *Mischocyttarus flavitarsus*. Representative spectra of metal accumulation in aculeate aculei are presented in Figure 2.7. Figure 2.8. shows the compositional imaging performed on *Paraponera clavata* with metals concentrated in the distal region of the aculeus, non-uniformly distributed. Potassium, chloride and phosphorous are scattered throughout the end of the aculeus, whereas zinc is restricted to the distal end of the aculeus.

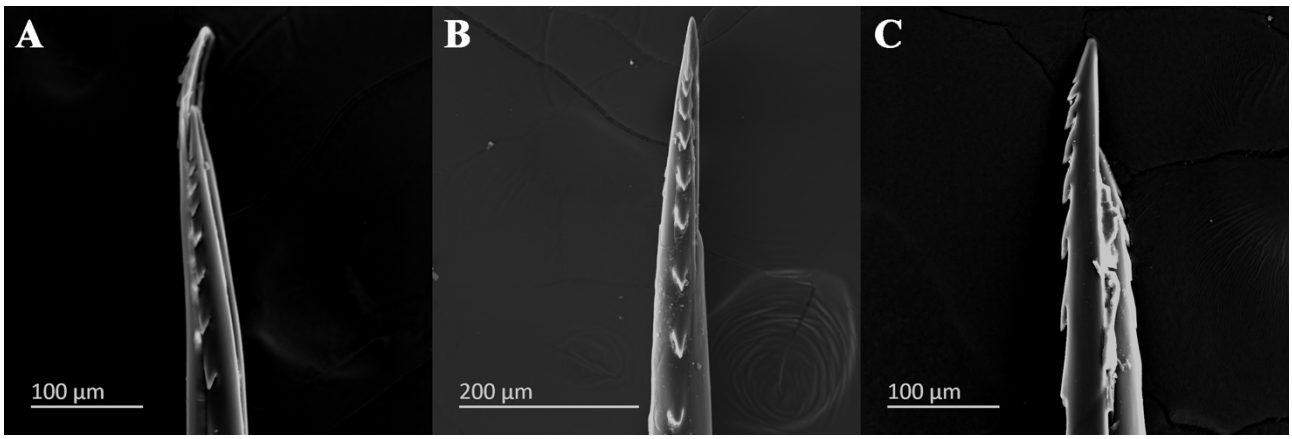

Figure 2.1. Scanning electron microscope (SEM) images of *Apis* genus.

(A) *Apis mellifera* (B) *Apis cerana* (C) *Apis dorsata*

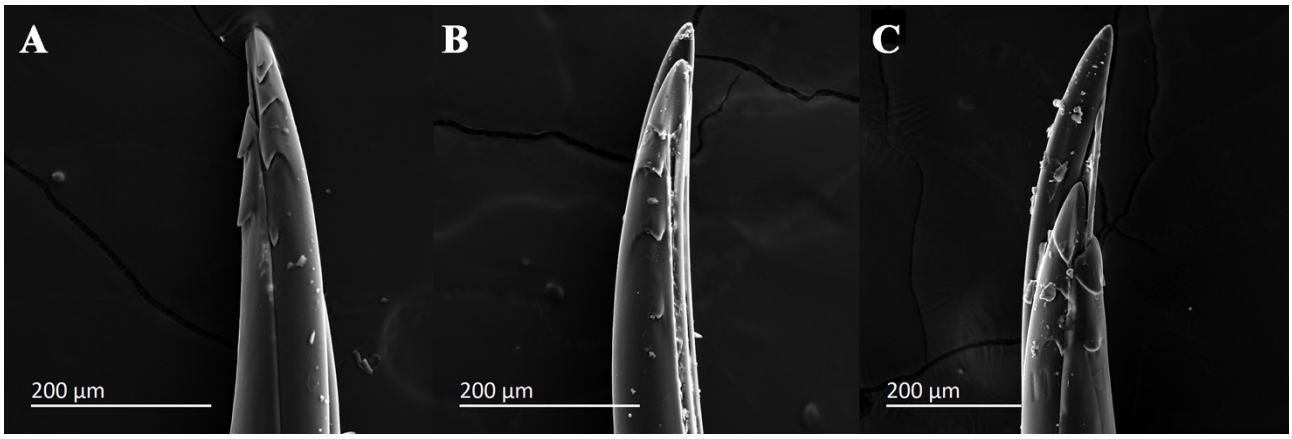

Figure 2.2. Scanning electron microscope (SEM) images of *Bombus* genus.

(A) *Bombus impatiens* (B) *Bombus huntii* (C) *Bombus sonorous*

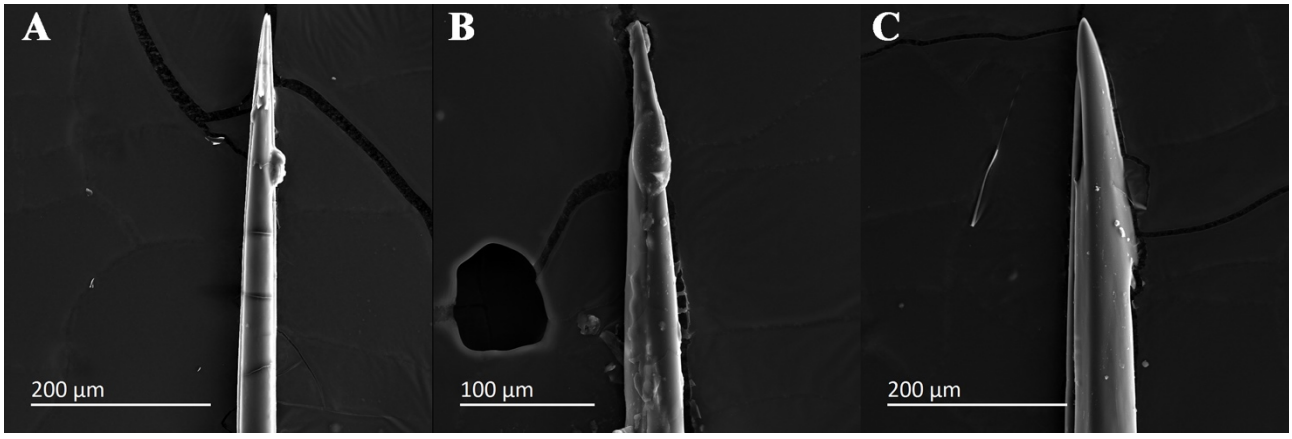

Figure 2.3. Scanning electron microscope (SEM) images of solitary bee species.

(A) *Centris rhodipus* (B) *Diadasia rinconis* (C) *Xylocopa rufa*

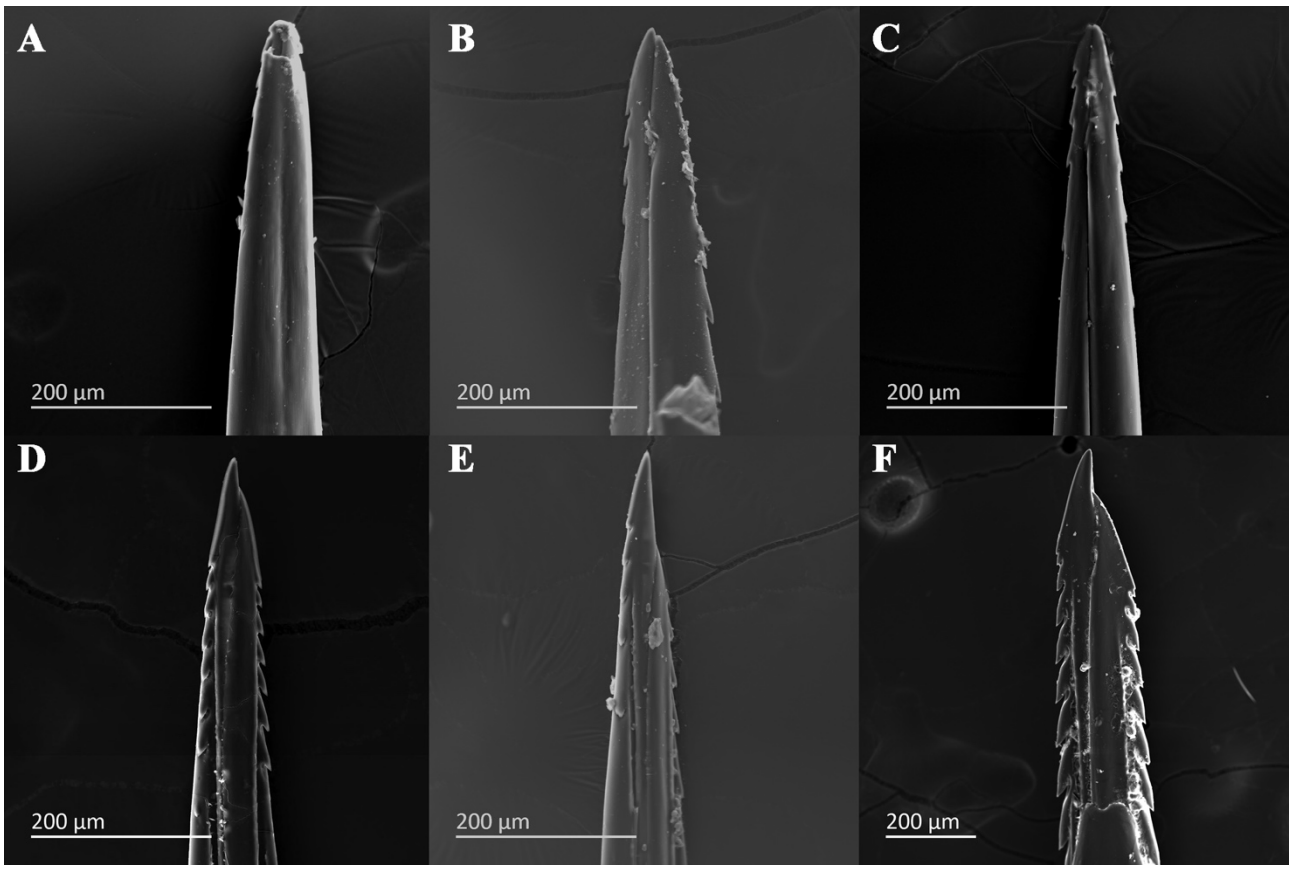

Figure 2.4. Scanning electron microscope (SEM) images of social wasp species.

(A) *Agelaia myrmecophila* (B) *Synoeca septrentrionalis* (C) *Polistes dorsalis* (D) *Vespa mandarinia* (E) *Dolichovespula maculata* (F) *Provespa anomala*

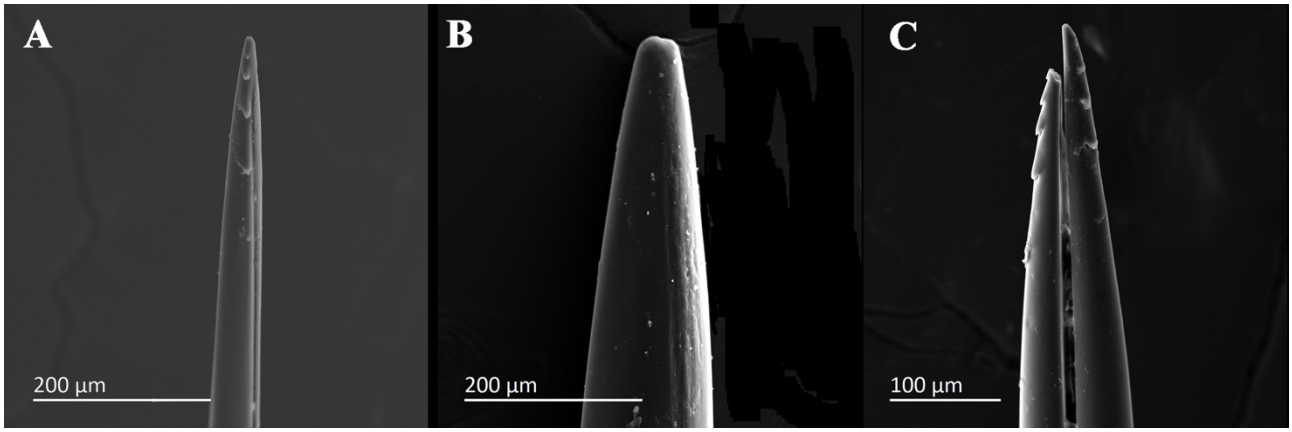

Figure 2.5. Scanning electron microscope (SEM) images of solitary wasp species.

(A) *Dasymutilla gloriosa* (B) *Scoliidae* spp. (C) *Stictia carolina*

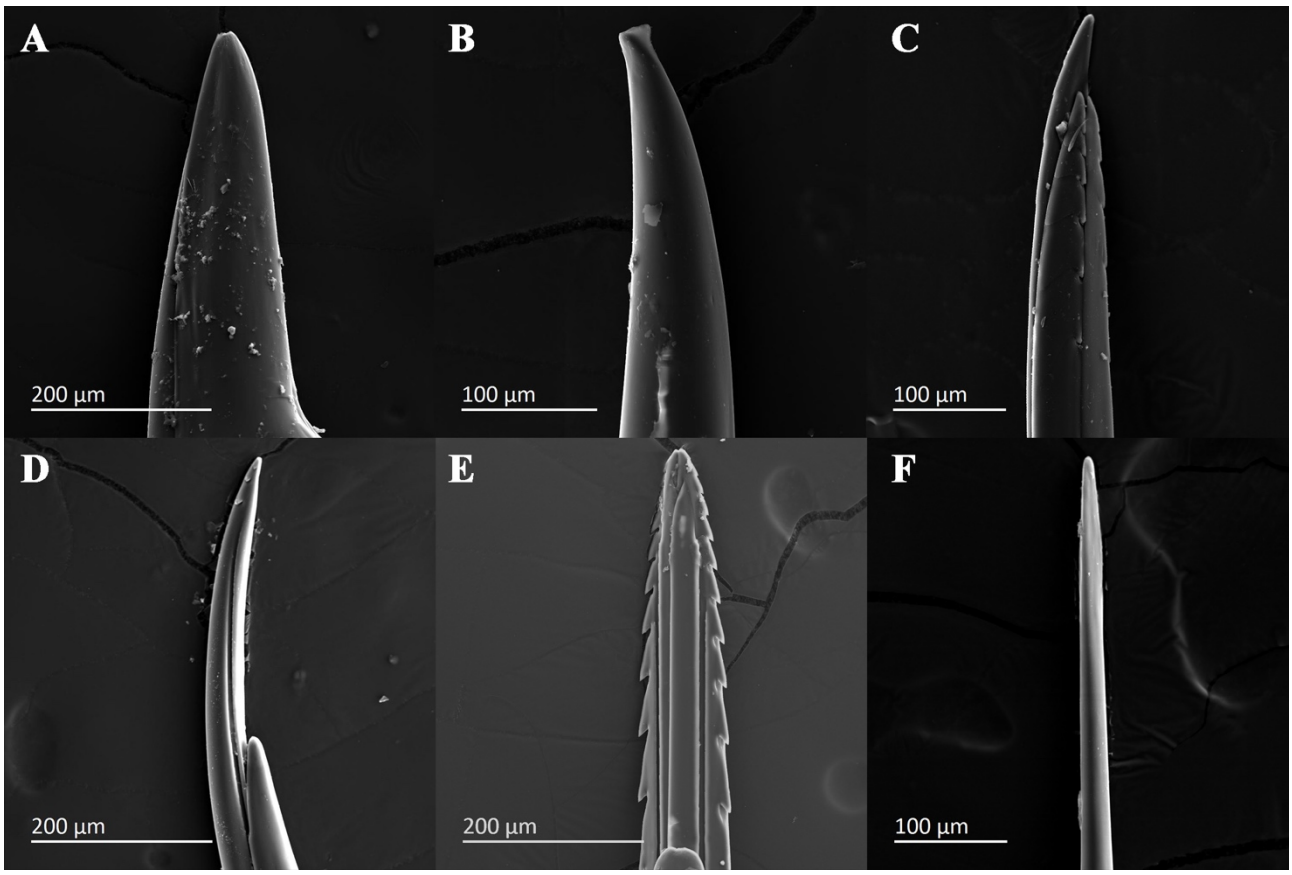

Figure 2.6. Scanning electron microscope (SEM) images of Formicidae species.

(A) *Odontoponera* spp. (B) *Myremcia gluosa* (C) *Paraponera clavata* (D) *Pogonomyrmex occidentalis* (E) *Ectatomma tuberculum* (F) *Gnaptogenys mordax*

2.3.3. Phylogenetic generalised least squares regression

We found that barbs present on the aculeus were not related to sociality (PGLS: t=1.28, df=1, p =0.20) or the presence of Ti (PGLS: t=-0.13, df=1, p=0.89), Fe (PGLS: t=0.89, df=1, p=0.37), Mn (PGLS: t=-0.27, df=1, p=0.79), Cu (PGLS: t=1.25, df=1, p=0.21). However, higher levels of Zn (PGLS: t=1.91, df=1, p=0.06) in the aculeus were marginally associated with barbs in the aculeus.

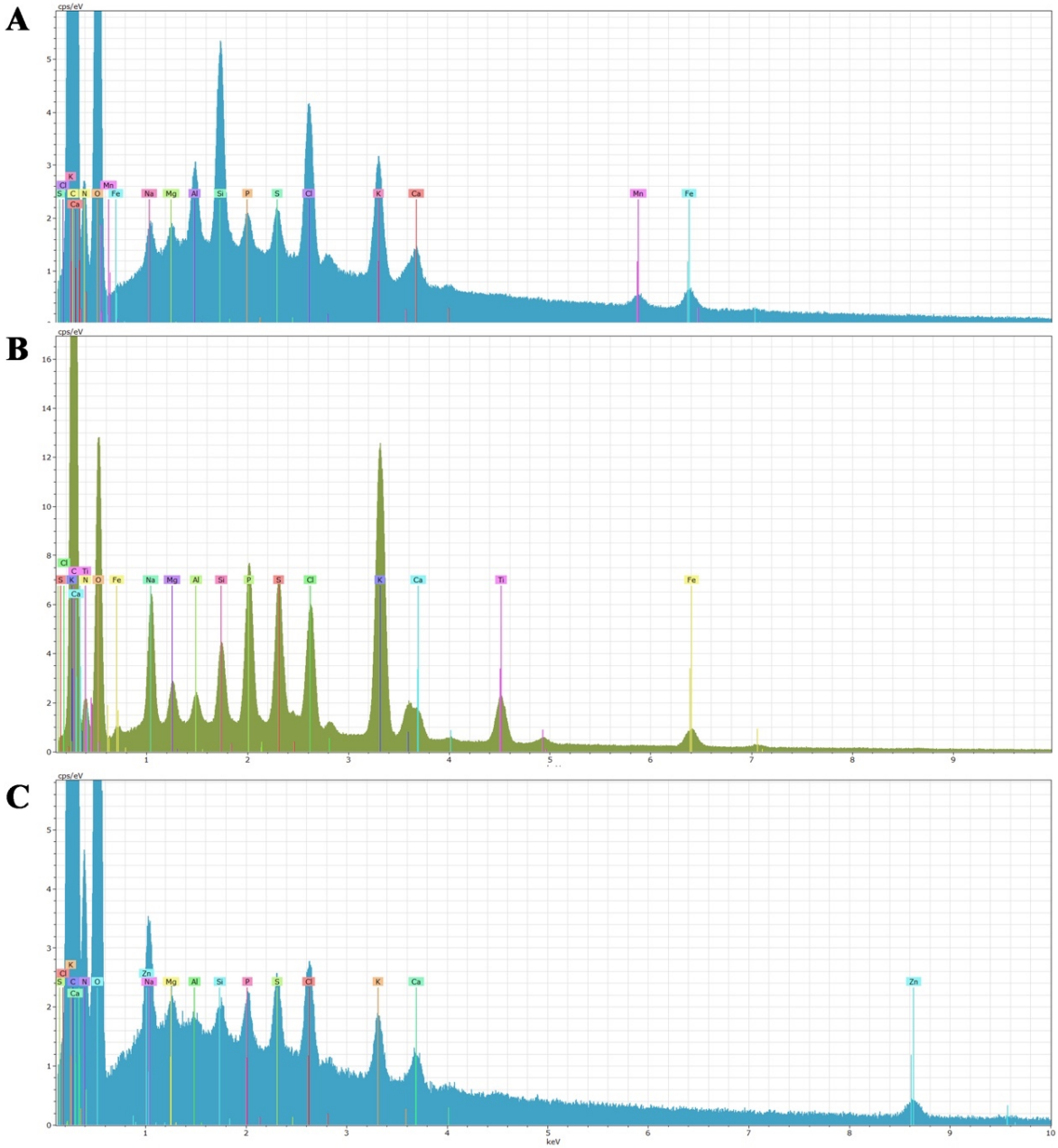

Figure 2.7. Representative EDS spectra

(A) *Bombus sonorous* (B) *Pogonomyrmex maricopa* (C) *Mischocyttarus flavitarsus*

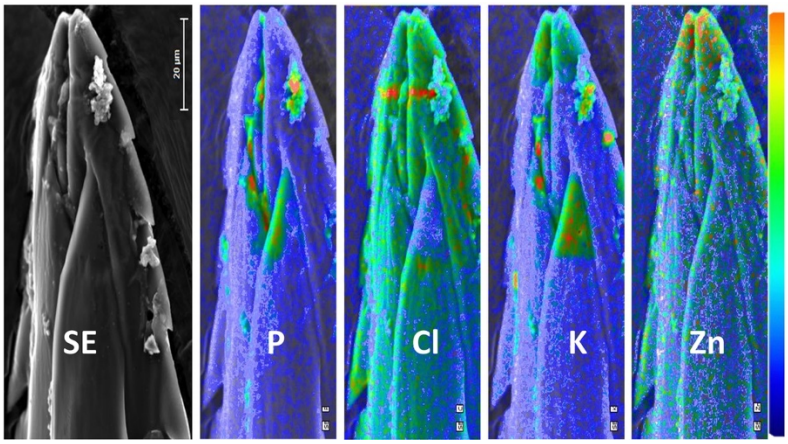

Figure 2.8. Compositional imaging of *Paraponera clavata* sting with 25° tilt and TOA 60°

Figure 2.9. Ancestral state reconstructions over branches for serrations where warmer colours represent a greater degree of serration in the stinger.

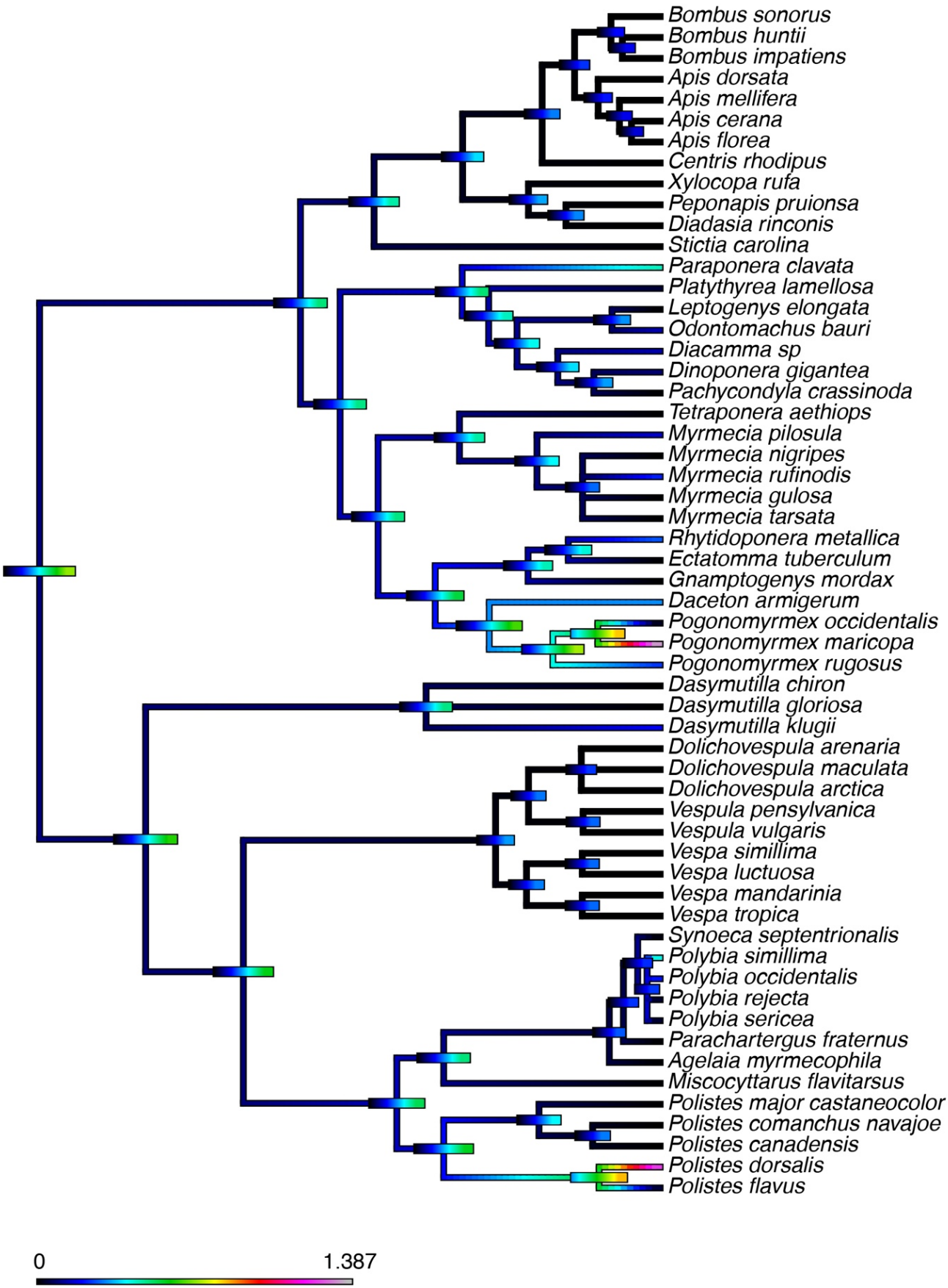

Figure 2.10. Ancestral state reconstructions over branches for zinc where warmer colours represent a higher concentration of zinc in the stinger.

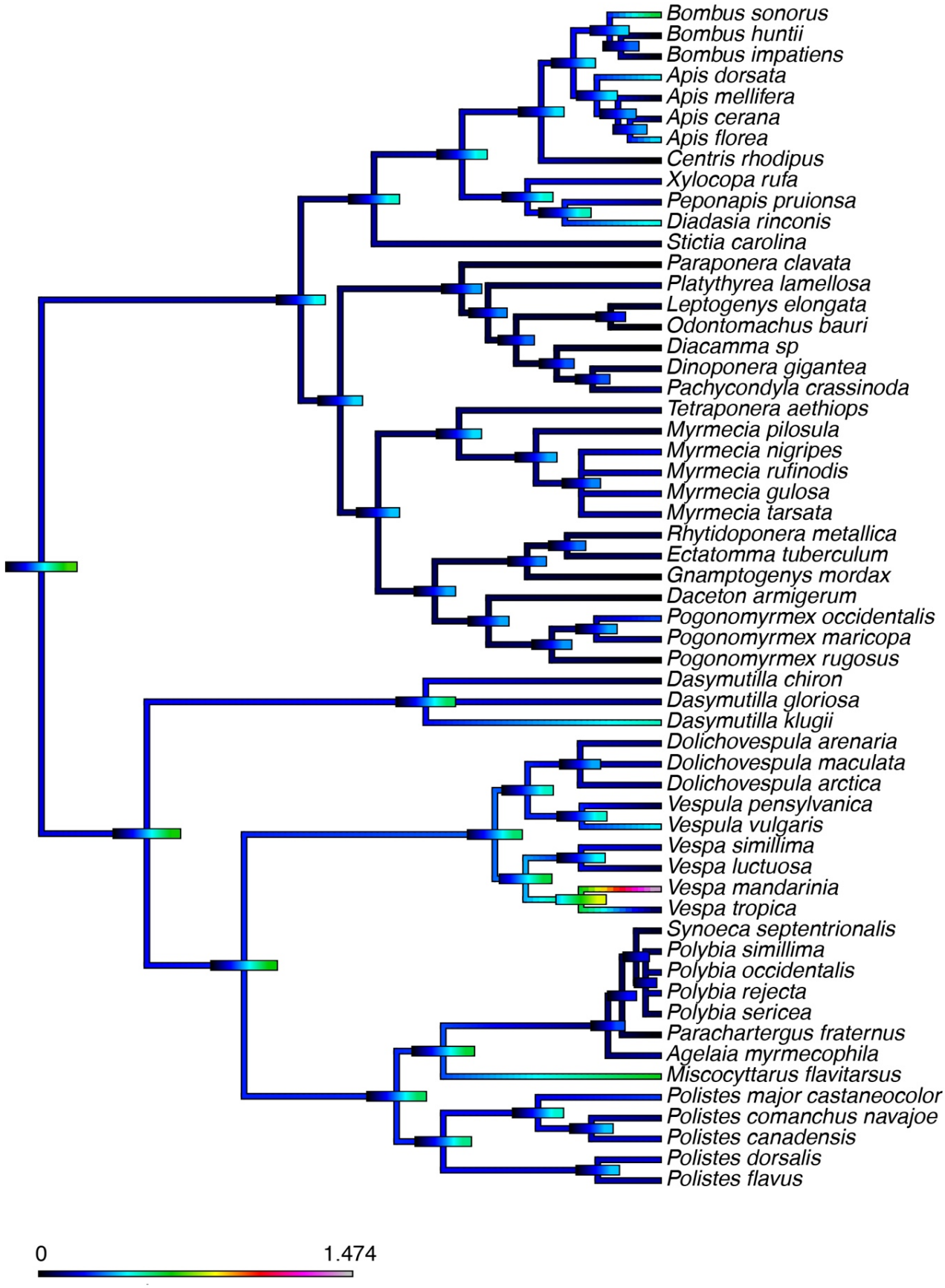

Figure 2.11. Ancestral state reconstructions over branches for iron where warmer colours represent a higher concentration of iron in the stinger.

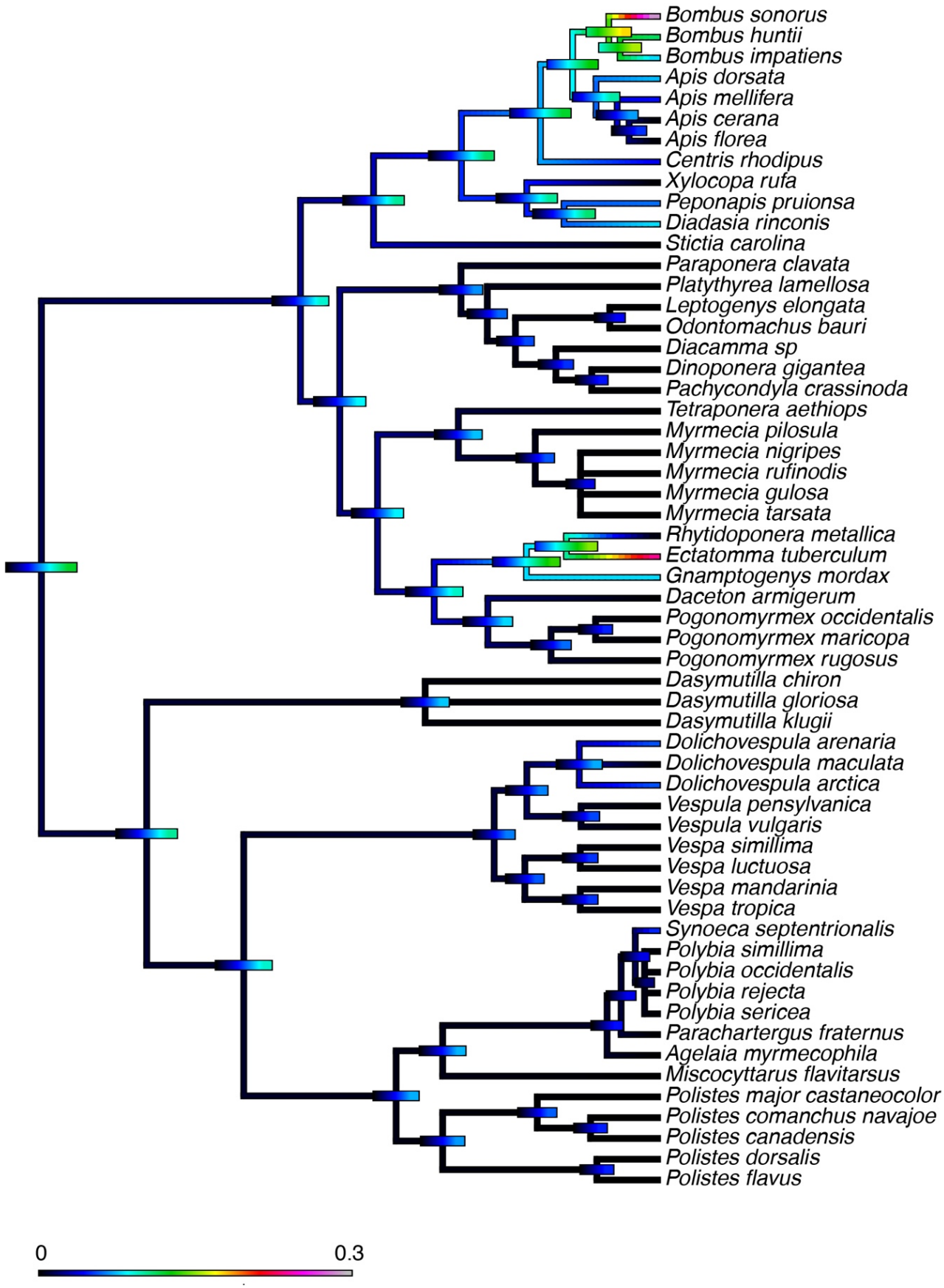

Figure 2.12. Ancestral state reconstructions over branches for manganese where warmer colours represent a higher concentration of manganese in the stinger.

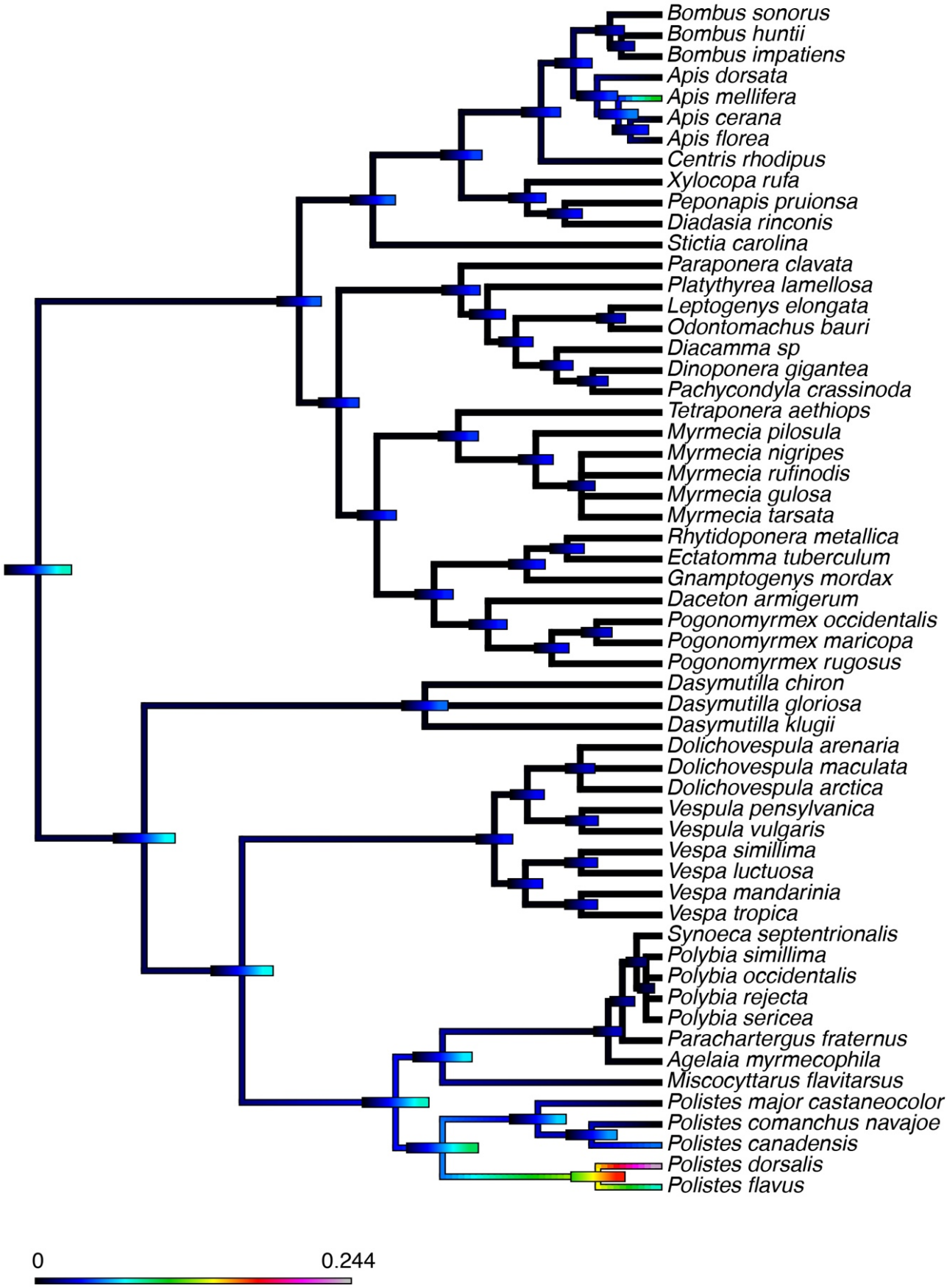

Figure 2.13. Ancestral state reconstructions over branches for copper where warmer colours represent a higher concentration of copper in the stinger.

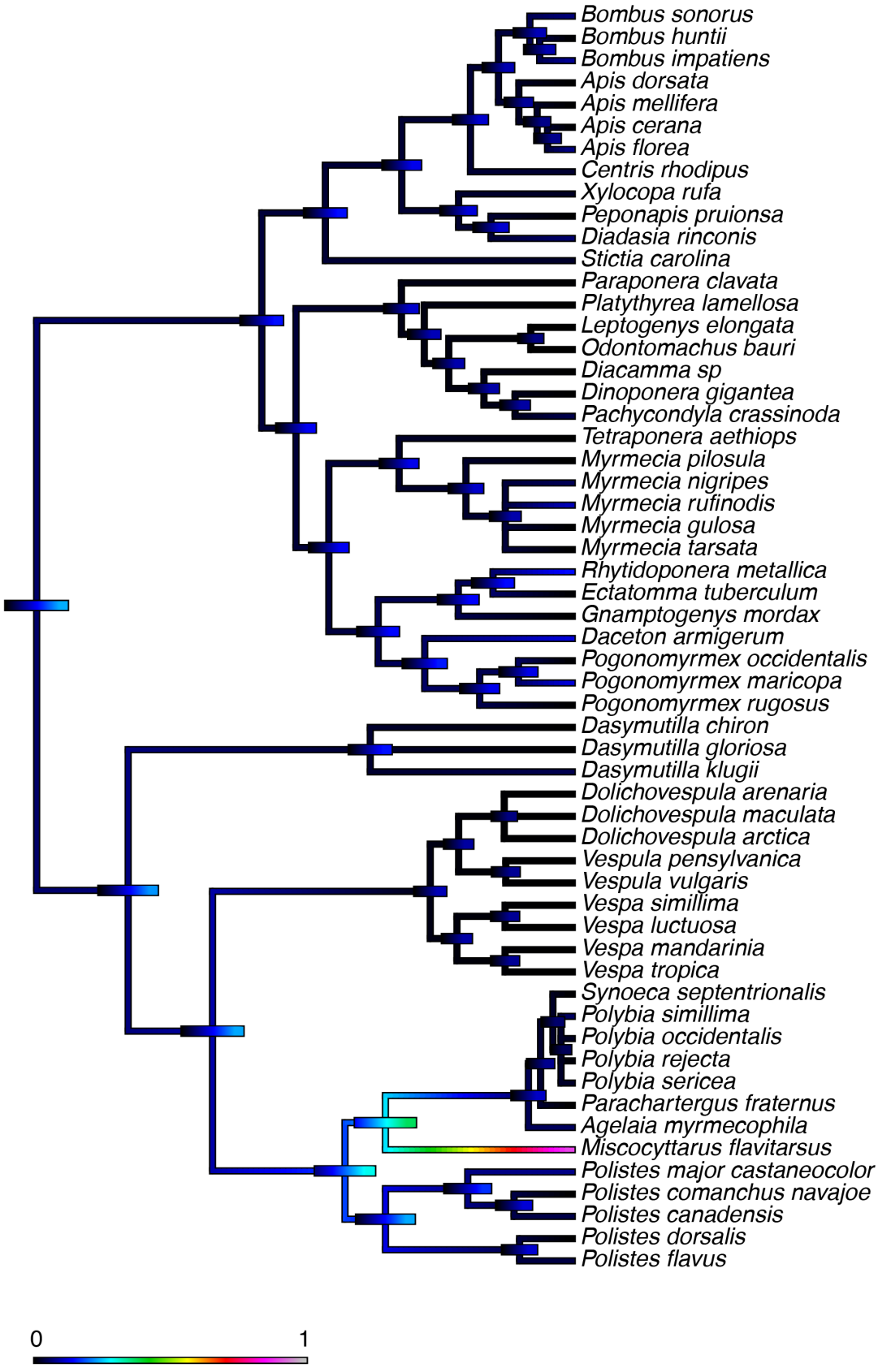

Figure 2.14. Ancestral state reconstructions over branches for titanium where warmer colours represent a higher concentration of titanium in the stinger.

Table 2.1. Presence of metals in the aculei of aculeates

Sociality	Family	Species	Voucher Code	Fe	Cu	Mn	Zn	Ti
Social	Apidae	*Apis mellifera*	USNMENT 01111826	1	1	1	-	-
	Apidae	*Apis cerana*	USNMENT 01110537	1	-	-	-	-
	Apidae	*Apis dorsata*	USNMENT 01080706	2	-	1	-	-
	Apidae	*Apis florea*	USNMENT 01111300	2	-	-	-	1
	Apidae	*Bombus impatiens*	USNMENT 01000423	-	-	1	-	1
	Apidae	*Bombus huntii*	USNMENT 01003891	-	-	1	-	-
	Apidae	*Bombus sonorus*	USNMENT 01033975	3	-	2	-	1
Solitary	Apidae	*Centris rhodipus*	USNMENT 01248229	-	-	1	-	1
	Apidae	*Diadasia rinconis*	USNMENT 01248238	2	-	1	-	1
	Apidae	*Peponapis pruinosa*	USNMENT 01248231	1	-	1	-	-
	Apidae	*Xylocopa rufa*	USNMENT 01248232	1	-	-	-	1
Social	Vespidae	*Agelaia myrmecophila*	USNMENT 01248201	1	-	-	-	1
	Vespidae	*Brachygastra mellifica*	USNMENT 01125241	-	-	-	-	-
	Vespidae	*Mischocyttarus flavitarsus*	USNMENT 01248210	3	-	-	-	3
	Vespidae	*Parachartergus fraternus*	USNMENT 01125240	-	-	-	-	-
	Vespidae	*Polistes canadensis*	USNMENT 01248205	1	1	-	-	1
	Vespidae	*Polistes comanchus navajoe*	USNMENT 01248212	1	-	-	-	-
	Vespidae	*Polistes dorsalis*	USNMENT 01125242	1	1	-	3	-

	Vespidae	*Polistes flavus*	USNMENT 01248206	1	1	-	1	1
	Vespidae	*Polistes major castaneocolor*	USNMENT 01248214	2	-	-	-	1
	Vespidae	*Polybia occidentalis*	USNMENT 01125244	1	-	-	1	1
	Vespidae	*Polybia rejecta*	USNMENT 01248215	1	-	-	-	-
	Vespidae	*Polybia sericea*	USNMENT 01248216	1	-	-	-	1
	Vespidae	*Polybia simillima*	USNMENT 01125243	1	-	-	2	1
	Vespidae	*Ropalidia stigma*	USNMENT 01248247	-	-	-	-	-
	Vespidae	*Dolichovespula maculata*	USNMENT 01248220	1	-	-	-	-
	Vespidae	*Dolichovespula arenaria*	USNMENT 01248219	1	-	1	-	-
	Vespidae	*Vespa luctuosa*	USNMENT 01248249	1	-	-	-	-
	Vespidae	*Vespa mandarinia*	USNMENT 01248221	3	-	-	-	-
	Vespidae	*Vespa simillima*	USNMENT 01248222	1	-	-	-	-
	Vespidae	*Vespa tropica*	USNMENT 01248253	1	-	-	-	-
	Vespidae	*Dolichovespula arctica*	USNMENT 01238524	1	-	1	-	-
	Vespidae	*Vespula pensylvanica*	USNMENT 01248224	1	-	-	-	-
	Vespidae	*Vespula vulgaris*	USNMENT 01248225	2	-	-	-	-
	Vespidae	*Synoeca septentrionalis*	USNMENT 01248218	1	-	1	-	-
	Vespidae	*Belonogaster juncea colonialis*	USNMENT 01248246	-	-	-	-	-
	Vespidae	*Provespa anomala*	USNMENT 01248248	-	-	-	-	-
Solitary	Mutillidae	*Dasymutilla chiron*	USNMENT 01248237	1	-	-	-	-

	Mutillidae	*Dasymutilla gloriosa*	USNMENT 01248228	1	-	-	-	-
	Mutillidae	*Dasymutilla klugii*	USNMENT 01248204	3	-	-	1	1
	Scoliidae	*Scoliidae sp.*	USNMENT 01248235	-	-	-	-	-
	Crabronidae	*Stictia carolina*	USNMENT 01248245	1	-	-	-	-
Eusocial	Formicidae	*Ectatomma tuberculatum*	USNMENT 01125478	1	-	1	-	-
	Formicidae	*Gnaptogenys mordax*	USNMENT 01125485	-	-	1	-	-
	Formicidae	*Rhytidoponera metallica*	USNMENT 01125467	1	-	-	1	1
	Formicidae	*Pogonomyrmex maricopa*	USNMENT 01125460	1	-	-	3	1
	Formicidae	*Pogonomyrmex occidentalis*	USNMENT 01125468	1	-	-	-	-
	Formicidae	*Pogonomyrmex rugosus*	USNMENT 01125487	-	-	-	1	-
	Formicidae	*Myrmecia gulosa*	USNMENT 01125476	1	-	-	-	-
	Formicidae	*Myrmecia nigripes*	USNMENT 01125463	1	-	-	-	1
	Formicidae	*Myrmecia pilosula*	USNMENT 01125934	1	-	-	-	-
	Formicidae	*Myrmecia rufinodis*	USNMENT 01125465	1	-	-	1	1
	Formicidae	*Myrmecia simillima*	USNMENT 01125470	-	-	-	-	
	Formicidae	*Myrmecia tarsata*	USNMENT 01125473	1	-	-	-	-
	Formicidae	*Daceton armigerum*	USNMENT 01125479	-	-	-	2	1
	Formicidae	*Paraponera clavata*	USNMENT 01125475	-	-	2	-	1
	Formicidae	*Pachycondyla crassinoda*	USNMENT 01125477	1	-	-	-	-
	Formicidae	*Dinoponera gigantea*	USNMENT 01125471	1	-	-	1	-

	Formicidae	*Leptogenys elongata*	USNMENT 01125488	1	-	-	-	-
	Formicidae	*Odontomachus bauri*	USNMENT 01125482	-	-	-	1	-
	Formicidae	*Diacamma sp.*	USNMENT 01125480	-	-	-	1	-
	Formicidae	*Platythyrea lamellosa*	USNMENT 01125486	1	-	-	1	-
	Formicidae	*Odontoponera sp.*	USNMENT 01125483	-	-	-	-	-
	Formicidae	*Streblognathus aethiopicus*	USNMENT 01125474	-	-	-	-	-
	Formicidae	*Tetraponera aethiopus*	USNMENT 01125484	1	-	-	-	-

Scale: 1 (<0.3%), 2 (0.31 – 0.6 weight %), 3 (0.61 – 1.5 weight %) or - (no metal detected)

2.4. Discussion

2.4.1. Morphology

Scanning electron micrographs showed striking differences in the morphologies of aculei across aculeates. The aculei in the genus *Apis* are all armed with backward sloping barbs (Figure 2.1.). These barbs are one of the reasons aculeus autotomy (the self-amputation of the aculeus) occurs in honeybees [26]. It has also been established that the muscles surrounding the aculeus in *Apis* species are significantly reduced, which contributes to aculeus autotomy [277]. The distance between each successive barb increases proximally in *Apis*. This arrangement has been postulated to assist in the firm penetration of each acute barb into the victim's body, thus ensuring that the aculeus cannot be retracted [278]. In contrast, species in the genus *Bombus* are able to retract their aculeus, which is reflected in the morphology of the barbs on the aculeus (Figure 2.2.). No solitary bee species show aculeus autotomy, which is consistent with the evidence provided herein by the lack of barbs (Figure 2.3.), although *Centris rhodipus* and *Diadasia rinconis* do have a short bump-like protrusion on the end of the stinger (Figure 2.3. a, b).

The aculeus of species examined from the family Vespidae showed a range of morphological variations from small barbs to large barbs (Figure 2.4.). Aculeus autotomy has also been found in genera of Vespidae [279]. The aculei from the solitary wasp species (Figure 2.5.) were similar to the stings of solitary bee species (Figure 2.3.) in lacking any substantial barbs. The aculei from Formicidae predominately lacked barbs (Figure 2.6.).

Aculeus autotomy is only known to occur in social Hymenoptera and has evolved independently on at least three separate occasions; bees of the genus *Apis*, ants of the genus *Pogonomyrmex*, and several species in the tropical wasp tribes Epiponini, Polistini, and Rhopalidiini [26, 280, 281]. Aculeus autotomy has previously been attributed to barb size [281]. The species found with barbs on the aculeus all belonged to groups that are known to present with aculeus autonomy with the exception of *Polistes* and *Mischocyttarus*, which have not been found to autotomise their aculeus. This suggests that barb presence and size may facilitate aculeus autotomy but are most likely not a requirement for this trait.

Barbs on the aculeus may be the ancestral trait in aculeates that has subsequently been secondarily lost in certain groups of aculeates, or they may have evolved separately on numerous occasions. If barbed aculei are the ancestral state this would follow that barbs

have been found in Hymenopterans outside aculeates; evidenced by the superfamilies Ichneumonoidea and Chalcidoidea [253, 282]. There have been no studies done on the morphology of the aculeus in Chrysidoidea. However, research focusing on aculeus morphology Chrysidoidea and Symphyta may further elucidate the evolutionary trajectory of this trait and whether it is the result of convergent evolution. The barbs have become more prominent in certain lineages of aculeates, most notably Apidae (Figure 2.1., 2.2.). However, as seen in certain Formicidae species (Figure 2.6.), barbs have been lost in some aculeate lineages. This loss of barbs on the aculeus in some lineages of aculeates is not significantly dependent on sociality but rather some other undetermined selection pressure.

2.4.2. Elemental composition and distribution

We also examined the presence of metals in the aculei. Transition metals were found in all families of aculeates studied; however, there were species within most of these families that do not show the presence of any detectable metals in the aculeus (Table 2.1.). As shown previously in parasitoids, there is considerable variation between taxa in whether or not they have high concentrations of metals associated with the aculeus [252]. This variation may be informative of the life history of the species, but further studies would be needed to confirm this.

Metals identified in the cuticle of the aculeus were iron, zinc, manganese, titanium and copper. Phylogenetic analyses found no strong relationship between the presence of iron, copper, manganese, or titanium and barbs on the aculeus, indicating a lack of direct evolutionary selection pressure between barbs present on the aculeus and concentrations of these metals (Figures 2.11. – 2.14.). However, there was a marginally positive relationship between barb presence and zinc (Figure 2.10.). Earlier research has shown that zinc enrichment in insects plays a functional role in the enhancing mechanical properties such as hardness, which may be why it has been linked to barb presence in this study [283-285]. Manganese enriched cuticles have been linked to increases in cuticle density and/or resistance to fracture [286]. Thus, indicating a role in species of this study, which is not co-selected for in relation to degree of barbing, but guided by unknown selection pressures. Iron and copper, while also quite common in the species sampled, are less studied and the particular effect these metals have on cuticle mechanical properties remains unclear.

Iron, zinc, manganese, and copper were found at 0.02 to 1.5 weight mass precent of the cuticle (Table 2.1.). This is a relatively minor fraction of the bulk composition and it has been

postulated that these metals are present at levels too low to affect changes in mechanical properties [287]. It seems likely that the low percentage of metals compared to those found in parasitoid species may be a result of the aculei not being required to be especially hardened or abrasion resistant, in comparison to wood-boring in gall-wasps or fig-wasps, for example. However, the cuticle is a complex matrix and still not well understood regarding its mechanical properties. The hardest insect material found was in the jaws of a jewel beetle larva that lacked the presence of metals and fed on wood. In seeming contradiction, the similarly dark coloured adult beetle mandibles contain the transition metal manganese, but were significantly softer [288]. This suggests that even though there are only low concentrations of metals present in the aculeus, it is not necessarily a softer material.

SEM-EDS point analyses reveal that metals occur almost exclusively around the tips and edges of the aculeus but rarely in the surrounding areas of the cuticle. Thus, while the overall metal content is low, the selective placement suggests an adaptive functional role. Consistent with this, enhanced amounts of zinc have also been found concentrated mainly in the ovipositor tips of several wood-boring wasps [252], several gall-parasitoid wasps [289], and the parasitic fig wasp *Apocrypta* [253]. In order to investigate the compositional differences further, compositional imaging was undertaken on the bullet ant *Paraponera clavata*. Again, the elemental distribution of the metals was non-uniform and concentrated in the distal region of the aculeus aculeus where the mechanical impact is expected to be highest. The tip of the *P. clavata* aculeus was enriched in chlorine, phosphorus, potassium, and zinc by factors of 6 to 100 times relative to bulk chitin and protein. This compositional imaging highlights that the presence of metals is concentrated in the tips of the aculei, which is to be expected with the tip bearing the brunt of any mechanical damage.

The forms of metal selectivity and sequestration is of interest in order to understand the mechanisms of metal binding in the organism; however, these processes are not well understood. There is limited evidence to support habitat, diet or phylogeny in determining why certain metals are selected [284, 285, 290-293]. Recently it has been postulated that genetic and cellular regulation may have the greatest control over which metals are utilised, rather that environmental metal availability [294]. The mechanisms for the biological sequestration of metals have not been well studied either. However, two potential binding mechanisms have been proposed: binding to amino acid side chains of cuticular proteins, or binding to catecholate ligands, which accumulate during cuticle sclerotization [292].

Nevertheless, our results do not allow us to distinguish these potential mechanisms and this is therefore an open question for future research. The link found between the presence of zinc and barb presence on the aculeus is interesting and could be useful as a model system in order to study the roles that these different metals play in the mechanical properties of the insect cuticle.

2.5. Conclusion

In conclusion, we used scanning electron imaging coupled with energy dispersive X-ray analysis to investigate the structure and metal composition of the aculeate aculeus. Through such methods, we show unique morphological features in the aculeus of aculeates, which are not associated with sociality. Our findings are also the first to show metal accumulation in ovipositors that are used exclusively for the delivery of venom. This research aids in understanding the evolution of these understudied insect venom systems and will have wide reaching effects pertaining to the evolution and ecology of stinging wasps, bees, and ants.

CHAPTER 3

Functional and Proteomic insights into Aculeata venoms: Evolutionary and Toxinological implications

Abstract

Solitary and social aculeates use their venom for different purposes. Solitary aculeates generally use their venom to paralyse and preserve prey without killing it, whereas, social aculeates use their venom in defence of their colony. These distinctive uses of venom suggest that the venom components and their functions are likely to differ based on the social behaviour of the aculeate. This study looks at a range of solitary and social species across Aculeata and uses a combination of proteomic and mass spectrometric techniques and activity assays in order to provide a foundation of information pertaining to the differences between solitary and social venoms. While there were many common components identified between species with differing social behaviours, major differences in the venoms could be seen in the presence and activity of active enzymes such as PLA_2 and serine protease, as well as the cytotoxic effects of the venom. Social aculeate venom was found to typically have a higher presence of peptides that cause damage and pain in the victim. Due to the nature of solitary venom it was expected to contain a unique array proteins and peptides, however, due to the scarcity of information present on solitary venoms, this could not be confirmed. Finally, this study provides the first look at a venom-gland transcriptome from the European honeybee, *Apis mellifera*.

3.1. Introduction

Aculeates present as a extremely diverse group of insects, containing a diversity of life histories and social behaviours (predatory, parasitic, pollinivorous). This group can be further categorised by whether the insect is solitary or social in lifestyle. Sociality was a major evolutionary transition, and is thought to have arisen multiple times in insects [295]. For sociality to be successful, a balance must be achieved between cooperation and conflict within the group. Although the selection mechanisms needed in order to achieve this balancing act are relatively well understood [296, 297], the venom biochemical mechanisms and consequences of eusociality remains unclear. As venom composition often correlates with the behaviour of the organism. It would seem likely that venom composition would also change with this important evolutionary transition. In order to fully understand how and if venom composition changes with the evolution of eusociality, it is necessary to compare venom changes in solitary and social species. Solitary and parasitic species of Aculeata use their venoms in order to paralyse and preserve their prey. Social species use their venom primarily for defence, some social species of wasp and ants have the additional function of using their venom for prey capture. As such venoms from social aculeates are important

defensive weapons, they have likely to have evolved in order to maximise pain and damage inflicted on predators, in addition to their ancestral predatory or parasitic functions.

Aculeate venoms are composed of a unique mixture of peptides, enzymes, and biogenic amines [57, 203, 298-300] and despite solitary species measurably outnumbering their social counterparts [301-303], the majority of venom research has focussed on social species, in particular the honeybee *Apis mellifera* [241]. The venom from species of Vespidae and Formicidae have also received attention, mostly due to their ability to cause allergic reactions in humans [99, 124, 162, 304]. However, most research has focussed on the characterisation and isolation of single molecules [66, 69-71, 73-76, 131], neglecting whole venom composition. This is important as evolutionary selection pressures 'see' the entire venom composition, not an individual toxin. Proteome studies are scant, with only a few species (mostly social) having been investigated [85-97]. The same neglect can be seen when looking at venom-gland specific transcriptomes, with less than a dozen sequenced [78, 79, 81, 82, 98], with the iconic honeybee *Apis mellifera* not amongst them. There are notably more transcriptomes from the whole body of the insect [83, 305-307], but this includes various whole-body proteins which are not relevant when looking for putative venom proteins. As such venom-gland transcriptomes are arguably more important when describing what toxins are expressed in the venom gland, which cannot be ascribed from a whole-body transcriptome. However, to our knowledge, there are no venom-gland transcriptome studies of bee species.

Aculeate venoms have also shown a proclivity to multifunctional activities that cause generalised pain, inflammation and in some cases anaphylactic shock [308]. In general, many of these reactions can be attributed in part to PLA_2 and serine protease enzymes. These enzymes have been found in both solitary and social venoms [99, 309], however, in many species the function has been implied rather than experimentally tested. Experimentally investigating these bioactivities will give a better understanding of species specific venom activity. Damage caused by aculeate venoms, is often the result of cytotoxic components. Such components have been reported to have potential anticancer effects, which has been extensively explored in bee venom [310-314], but neglected in the majority of other aculeate species with few exceptions [315-318]. Further exploring the cytotoxic abilities of venoms, will be instrumental in developing a roadmap in which to discover novel anticancer drugs.

Due to ever evolving technological advances, it has become increasingly easier to unravel the venom composition using combined approaches such as transcriptomics and proteomics techniques. This study lays the ground work for further deep comparative analysis between social and solitary species, including providing the first venom-gland transcriptome of any bee species; *Apis mellifera*. It is also the first study to provide a large-scale comparison of social and solitary Aculeata venom using a variety of transcriptomic, proteomic and bioactivity analyses.

3.2. Materials and methods

3.2.1. Taxonomic Selection

The species included in this study are outlined in Table 3.1. These species were selected in order to provide coverage that is both phylogenetically diverse across the families in Aculeata and to include both solitary and social species. The venom has been predominately collected via dissection of the venom glands. However, some in species in order to acquire the venom they needed to be milked as described in Schmidt et al. [216].

3.2.2. Proteomics

3.2.2.1. SDS-PAGE

One-Dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), shotgun tandem-mass spectrometry (MS/MS) were carried out as previously described [319-321].

3.2.2.2. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analyses HPLC analysis of 25 µg crude venom was performed on a Nexera system (Shimadzu) using a Zorbax 300SB C18, 3.5µm column (2.1 × 100 mm, Agilent) at a flow rate of 300 µl/min. The gradients adopted were: 2–40% Buffer B (90% acetonitrile) over 35 min, 40–98% Buffer B in 2 min, and left stable at 98% Buffer B for 2 min. Buffer A was 0.1% formic acid in water. The HPLC was directly connected to a DuoSprayTM ion source (ESI SCIEX) - TripleTOF 5600, operated in po- sitive ion acquisition mode. Data were acquired for 46 min over the m/z range 350–2000 Da with a cycle time of 0.5 sec. Raw results were analysed in Analyst® (SCIEX) and protein mass picks have been manually reconstructed. (Appendix I Supplementary Figures 3.1. – 3.9.).

Table 3.1. Taxonomic sampling of species investigated

Group	Family	Subfamily	Species
Social Bees	Apidae	Apinae	*Apis andreniformis*
	Apidae	Apinae	*Apis cerana*
	Apidae	Apinae	*Apis dorsata*
	Apidae	Apinae	*Apis florea*
	Apidae	Apinae	*Apis koschnikova*
	Apidae	Apinae	*Apis mellifera (*African and European*)*
	Apidae	Apinae	*Bombus impatiens*
	Apidae	Apinae	*Bombus huntii*
	Apidae	Apinae	*Bombus sonorus*
Solitary Bees	Apidae	Apinae	*Centris aethyctera*
	Apidae	Apinae	*Centris rhodipus*
	Apidae	Apinae	*Diadasia rinconis*
	Apidae	Apinae	*Peponapis pruinosa*
	Apidae	Apinae	*Xenoglossa angustior*
	Apidae	Xylocopinae	*Xylocopa rufa*
	Apidae	Xylocopinae	*Xylocopa californica*
	Apidae	Xylocopinae	*Xylocopa veripuncta*
	Colletidae	Diphaglossinae	*Crawfordapis sp*
	Halictidae	Halictinae	*Lasioglossum sp*
Social Wasps	Vespidae	Polistinae	*Agelaia myrmecophila*
	Vespidae	Polistinae	*Belonogaster juncea colonialis*
	Vespidae	Polistinae	*Brachygastra mellifica*
	Vespidae	Polistinae	*Mischocyttarus flavitarsus*
	Vespidae	Polistinae	*Parachartergus fraternus*
	Vespidae	Polistinae	*Polistes canadensis*
	Vespidae	Polistinae	*Polistes comanchus navajoe*
	Vespidae	Polistinae	*Polistes dorsalis*
	Vespidae	Polistinae	*Polistes flavus*
	Vespidae	Polistinae	*Polistes major castaneocolor*
	Vespidae	Polistinae	*Polybia rejecta*
	Vespidae	Polistinae	*Polybia sericea*
	Vespidae	Polistinae	*Polybia simillima*
	Vespidae	Polistinae	*Ropalidia sp*
	Vespidae	Polistinae	*Synoeca septentrionalis*
	Vespidae	Vespinae	*Dolichovespula arctica*
	Vespidae	Vespinae	*Dolichovespula arenaria*
	Vespidae	Vespinae	*Dolichovespula maculata*
	Vespidae	Vespinae	*Vespa luctuosa*
	Vespidae	Vespinae	*Vespa mandarinia*
	Vespidae	Vespinae	*Vespa simillima*
	Vespidae	Vespinae	*Vespa tropica*
	Vespidae	Vespinae	*Vespula pensylvanica*
	Vespidae	Vespinae	*Vespula vulgaris*
	Vespidae	Vespinae	*Provespa sp*
Solitary Wasps	Mutillidae	Sphaeropthalminae	*Dasymutilla chiron*
	Mutillidae	Sphaeropthalminae	*Dasymutilla gloriosa*

	Mutillidae	Sphaeropthalminae	*Dasymutilla klugii*
	Scoliidae	Scoliinae	*Scoliidae sp.*
	Crabronidae	Bembicinae	*Stictia sp.*
Ants	Formicidae	Ectatomminae	*Ectatomma quadridens*
	Formicidae	Ectatomminae	*Ectatomma tuberculatum*
	Formicidae	Ectatomminae	*Gnaptogenys sp.*
	Formicidae	Ectatomminae	*Rhytidoponera metallica*
	Formicidae	Mymicinae	*Pogonomyrmex maricopa*
	Formicidae	Mymicinae	*Pogonomyrmex occidentalis*
	Formicidae	Mymicinae	*Pogonomyrmex rugosus*
	Formicidae	Myrmeciinae	*Myrmecia browningii*
	Formicidae	Myrmeciinae	*Myrmecia gulosa*
	Formicidae	Myrmeciinae	*Myrmecia nigripes*
	Formicidae	Myrmeciinae	*Myrmecia pilosula*
	Formicidae	Myrmeciinae	*Myrmecia rufinodis*
	Formicidae	Myrmeciinae	*Myrmecia simillima*
	Formicidae	Myrmeciinae	*Myrmecia tarsata*
	Formicidae	Myrmicinae	*Daceton sp.*
	Formicidae	Ponerinae	*Diacamma sp.*
	Formicidae	Ponerinae	*Dinoponera gigantea*
	Formicidae	Ponerinae	*Euponera sennaarensis*
	Formicidae	Ponerinae	*Leptogenys sp.*
	Formicidae	Ponerinae	*Neoponera villosa*
	Formicidae	Ponerinae	*Odontomachus sp.*
	Formicidae	Ponerinae	*Opthalmopone sp.*
	Formicidae	Ponerinae	*Megaponera analis*
	Formicidae	Ponerinae	*Pachycondyla crassinoda*
	Formicidae	Ponerinae	*Paltothyreus tarsatus*
	Formicidae	Ponerinae	*Platythyrea lamellosa*
	Formicidae	Ponerinae	*Platythyrea strigulosa*
	Formicidae	Ponerinae	*Streblognathus aaethiopicus*
	Formicidae	Ponerinae	*Termitopone commutata*
	Formicidae	Ponerinae	*Termitopone commutata* (Queen)

3.2.3. Transcriptomics

3.2.3.1. RNA Extraction and Library Preparation

Apis mellifera species were sampled from EcoSciences Precinct, University of Queensland, Australia. Total RNA (tRNA) was extracted from venom glands by standard TRIzol protocol (ThermoFisher, Waltham, MA, USA). The RNA sample was submitted to the University of Queensland Institute for Molecular Bioscience Sequencing Facility for library preparation and sequencing. A paired end library with 180 bp insert size was constructed using the Illumina TruSeq-3 Stranded mRNA kit and sequenced on an Illumina NextSeq using a 300 cycle (2 x 150 bp) mid output run.

3.2.3.2. Sequence data pre-processing and transcriptome assembly

The resulting reads were trimmed using Trimmomatic v0.35 [322] to remove adapter sequences and low-quality reads. Window function-based quality trimming was performed using a window size of 4 and a window quality of 20 and sequences with a resulting length of <100 bp after trimming were removed. The trimmed reads were *de novo* assembled into contigs by Trinity v2.4.0 [323] using default parameters. Following assembly by Trinity, the trimmed paired reads from each tissue was mapped back to the assembly using Bowtie v2.2.6 [324] and expression values estimated as transcripts per kilobase million (TPM) using RSEM v1.2.31 [325].

3.2.3.3. Transcriptome Annotation

The *de novo* assemblies were concatenated and searched against reference toxin sequences obtained from UniProt using BLAST version 2.7.1 [326, 327]. CD-HIT v4.7 was used to cluster the sequences and remove duplicates [328, 329]. The remaining contigs that did not contain complete coding sequences were removed.

3.2.4. Bioactivity Activity Testing

3.2.4.1. Enzymatic activity studies

A Thermo Scientific™ Fluoroskan AscentTM Microplate Fluorometer was employed to test variation in enzymatic activity. For assessing the PLA_2 activity a fluorescence substrate assay was used (E10217 EnzChek® Phospholipase A_2 Assay Kit, ThermoFisher Scientific). Venom solution (0.1 µg in dry venom weight) was brought up to 12.5 µl in PLA_2 reaction buffer (250 mM Tris–HCL, 500 mM NaCl, 5 mM CaCl2, pH 8.9) and plated out in triplicates on a 384 well plate. Triplicates were measured by adding 12.5 µl quenched 1 mM EnzChek® Phospholipase A_2 substrate per well (total volume 25 µl/well) over 100 cycles at an excitation

of 485 nm and emission of 520 nm, using a Fluoroskan Ascent (ThermoFisher Scientific). The negative control consisted of PLA_2 reaction buffer and substrate only.

For testing on RDES substrate (Fluorogenic Peptide Substrate, R & D systems Cat#s ES0011, Minneapolis, Minnesota), 10ul of 0.05 µg/µl venom stock was plated in triplicate on a 384-well black plate and measured by adding 90 µl quenched fluorescent substrate per well. The substrate concentration was 10 µl of each substrate stock solution dissolved into 4.990ml of enzyme buffer (150 mM NaCl and 50 mM Tris-HCl pH 7.4). Fluorescence was monitored over 400 min or until activity ceased. Excitation was at 390 nm and emission was at 460 nm for substrate ES011. The machine was programmed to shake the plate for three sec before each reading to maintain homogeneity in the wells. Relative enzymatic activity was calculated as an increase in absorbance corresponding to the cleavage of the fluorescent group. Finally, the raw data were normalized to meet analysis assumptions and processed with GraphPad Prism 7.0.

3.2.4.2. Cytotoxicity studies

The effect of each venom was assessed on human neonatal foreskin fibroblast (NFF) and malignant melanoma (MM96L) cell lines, supplied by QIMR Berghofer Medical Research institute. Venom mediated cytotoxicity is often responsible for the degradation and destruction of skin and connective tissue. Therefore, the chosen cell lines were deemed appropriate. Cell lines were maintained in supplemented with 1% penicillin streptomycin and foetal calf serum (FCS), 10% FCS for NFF and 5% FCS for MM96L. Cells were split 24 h prior to the experiment (for up to 25 passages for MM96L and 10 passages for NFF) using 0.25% trypsin and seeded in 96 well flat bottom plates at a density of 5000 and 2500 cells/well for NFF and MM96L cells, respectively. Plates were incubated overnight at 37 °C in a 5% CO_2 95% humidified environment prior to treatment. Cell viability was evaluated using colorimetric MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma Aldrich M5655, Sydney, NSW, Australia) assays. Venom was added to cells at 5 g and 0.5 g protein amounts and followed by a 48 h incubation period. MTT was added at a concentration of 5 mg/mL per well. An amount of 0.1% sodium dodecyl sulphate (SDS) was used as a positive control to achieve 100% toxicity, and the protocol was followed according to the manufacturer's description. The absorbance was read at 570 nm on the PowerWave XS2 plate reader (Bio Tek Instruments, Winooski, VT, USA), using Gen5 software. Two independent experiments were conducted with a minimum of three replicates per treatment. Cell viability readings were normalized as a percent of untreated control cells, and viability

expressed as a percentage of toxicity ± standard error of the mean (SEM). The relationship between venom dose and cytotoxic response was calculated via area under the curve (AUC) analysis, using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) (Appendix I Supplementary Tables 3.1., 3.2.).

3.3. Results

3.3.1. Transcriptome

Multiple toxin types were sequenced from the *Apis mellifera* venom gland libraries. Analysis of the venom gland revealed the presence of various transcripts with significant homology to previously characterised venom toxins from hymenopterans (Table 3.2.). Transcripts sequenced were acid phosphatase, apamin, carboxylesterase, hyaluronidase, icarapin, mast cell degranulating peptide (MCD), melittin, phospholipase A2 (PLA_2), serine protease and venom allergen. Alignment of the translated amino acid sequences revealed little variation in the molecular structure of the transcripts for most toxin types.

The acid phosphatase, hyaluronidase, melittin and venom allergen transcripts were identical to the published protein sequences, with no evidence of duplication or diversification occurring. Apamin, carboxylesterase, icarapin, MCD, PLA_2, serine protease and tertiapin transcripts were also conserved with ancestral cysteine numbers and spacing preserved and with minimal sequence variation relative to published sequences (Figure 3.1.).

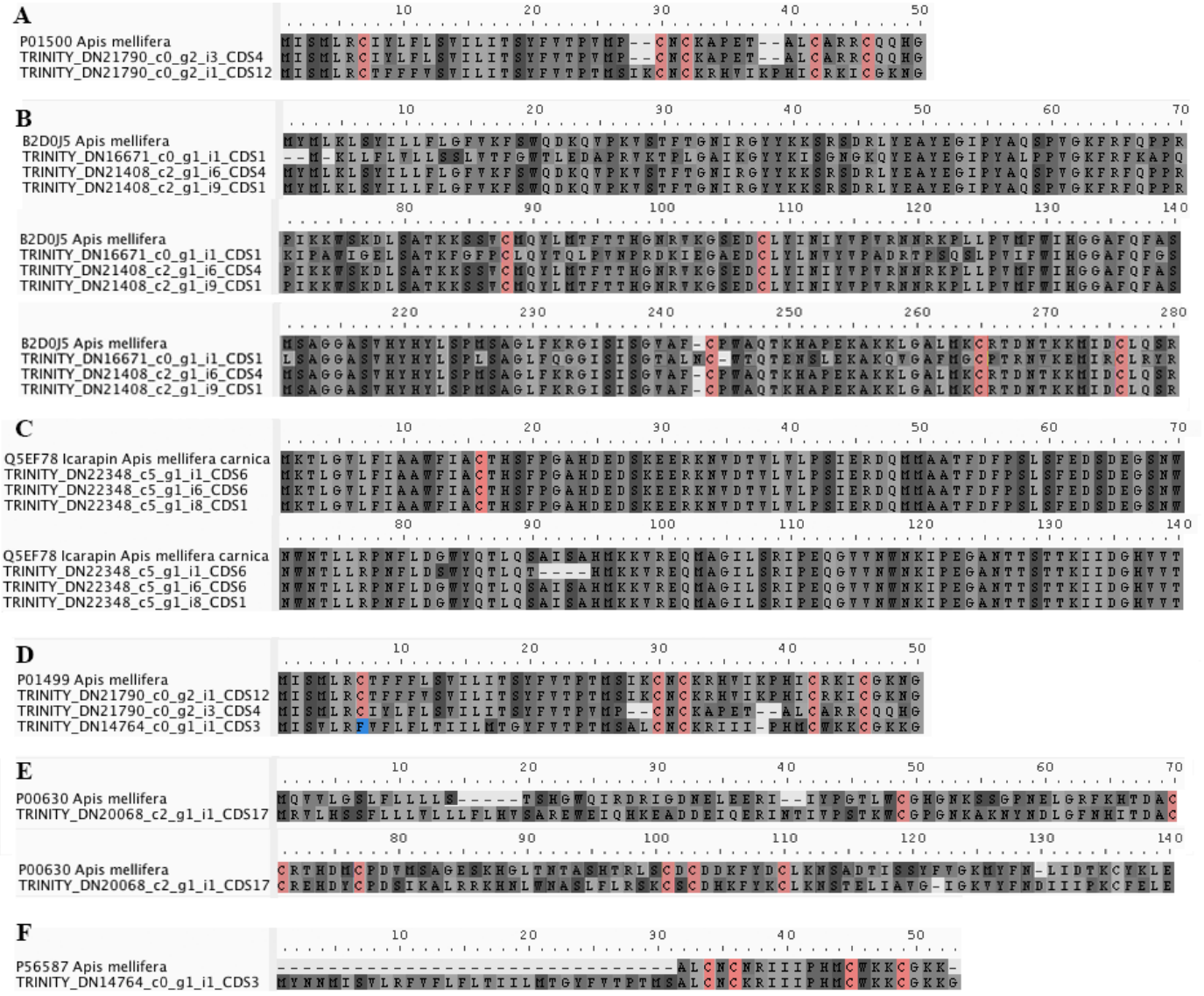

Figure 3.1. Excerpts of mature peptide sequences showing the cysteine sites highlighted in colour.

A) Apamin, B) Carboxylesterase, C) Icarapin, D) Mast cell degranulating peptide, E) Phospholipase A_2, F) Tertiapin

Table 3.2. Characterised bioactivities from venoms

Toxin type	**Bioactivities**
Acid Phosphatase	Hydrolysis of phosphomonoesters at acidic pHs
Apamin	Neurotoxin
Carboxylesterase	Allergen
Hyaluronidase	Venom spreading factor
Icarapin	Allergen
Mast Cell Degranulating Peptides	Degranulation of mast cells
Melittin	Hemolytic, cytotoxic, algogen, cardiotoxic
PLA2	Hydrolysis of lecitins
Serine Protease	Allergen, hemostasis, spreading factor
Tertiapin	Neurotoxin
Venom allergen	Allergen

Table 3.3. Putative Toxins identified using LC-MS/MS searched against uniprot database

Toxin Name	Social Species	Solitary Species
Hyaluronidase	Apis mellifera (European), Apis andreniformis, Apis cerana, Apis dorsata, Bombus huntii, Bombus impatiens, Dolichovespula arenaria, Megapolistes, Polybia rejecta, Vespa mandarinia, Vespula vulgaris, Platythyrea strigulosa, Streblognathus aethiopicus	Diadasia rinconis, Centris aethyctera, Xylocopa californica, Xylocopa veripuncta
Phospholipase A_2	Apis mellifera (Africanised and European), Apis andreniformis, Apis cerana, Apis dorsata	
Phospholipase A_1	Agelaia myrmecophila, Brachygastra mellifica, Dolichovespula arenaria, Mischocyttarus flavitarsus, Megapolistes, Polistes canadensis, Polistes dorsalis, Polistes flavus, Polybia sericea, Synoeca septentrionalis, Vespa luctuosa, Vespa mandarinia, Vespula pensylvanica, Vespula vulgaris, Pachycondyla crassinoda	Dasymutilla chiron
Venom acid phosphatase	Apis mellifera (Africanised and European)	Centris aethyctera
Venom carboxylesterase	Apis mellifera (European), Apis cerana, Apis dorsata, Apis florea	
Serine Protease	Apis mellifera (Africanised and European), Bombus huntii, Bombus impatiens	Peponapis pruinose, Dasymutilla klugii
Venom dipeptidyl peptidase	Brachygastra mellifica, Vespula pensylvanica, Vespula vulgaris	
Melittin	Apis mellifera (Africanised and European), Apis andreniformis, Apis cerana, Apis dorsata, Apis florea, Apis koschnikova, Bombus huntii, Bombus impatiens, Bombus sonorous, Vespa mandarinia, Dinoponera gigantea	
Icarapin-like	Apis mellifera (Africanised and European), Apis cerana, Apis dorsata	
Api m 6	Apis mellifera (Africanised and European)	
Venom allergen 5	Agelaia myrmecophila, Dolichovespula arenaria, Megapolistes, Polistes comanchus navajoe, Polistes flavus, Polistes major castaneocolor, Polybia simillima, Synoeca septentrionalis, Vespa luctuosa, Vespa mandarinia, Vespa simillima, Vespula pensylvanica, Vespula vulgaris , Pachycondyla crassinoda	
Apamin	Apis mellifera (Africanised and European), Apis florea, Apis koschnikova	
Bradykinin	Megapolistes, Polistes dorsalis, Polistes flavus, Polybia simillima, Vespula vulgaris	
Vespakinin	Polistes comanchus navajoe, Vespa mandarinia, Vespula pensylvanica, Vespula vulgaris	
Wasp kinin	Agelaia myrmecophila, Belonogaster juncea colonialis, Brachygastra mellifica, Dolichovespula arenaria, Polistes major castaneocolor, Polybia simillima, Synoeca septentrionalis, Parachartergus fraternus	
Mastoparan	Polistes major castaneocolor, Vespa luctuosa, Vespa mandarinia, Vespa simillima	
Pilosulin	Neoponera villosa	
Ectatomin	Ectatomma tuberculatum, Myrmecia rufinodis	
Megascoliakinin	Mischocyttarus flavitarsus	
Bombolitin	Apis mellifera (Africanised and European), Apis andreniformis, Apis cerana, Apis florea, Bombus huntii, Bombus impatiens, Bombus sonorus	

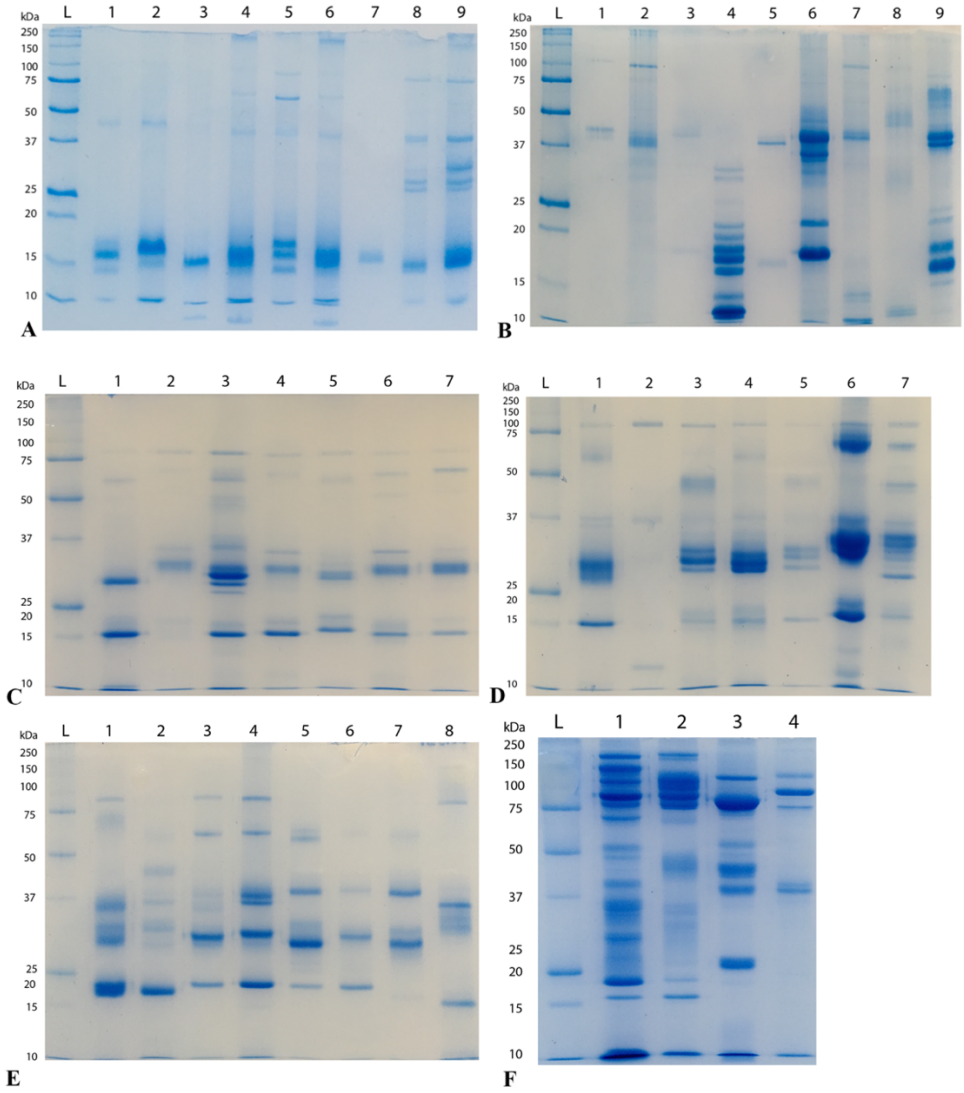

Figure 3.2. 1D SDS-PAGE Bees and Wasps A) Social Bees (reduced); 1 = *Apis mellifera* (European); 2 = *Apis mellifera* (Africanised); 3 = *Apis andreniformis*; 4 = *Apis cerana*; 5 = *Apis dorsata*; 6 = *Apis florea*; 7 = *Apis koschnikoa*; 8 = *Bombus huntii*; 9 = *Bombus impatiens*

B) Solitary Bees (reduced); 1 = *Centris aethycetra*; 2 = *Centris rhodipus*; 3 = *Diadasia rinconis*; 4 = *Peponapis pruinosa*; 5 = *Xylocopa rufa*; *6* = *Xylocopa californica*; 7 = *Crawfordapis*; 8 = *Lasioglossum kinabalueuse*; 9 = *Xylocopa veripuncta*

C) Epiponini wasps (reduced); 1 = *Agelaia myrmecophila*; 2 = *Brachygastra mellifica*; 3 = *Polistes flavus*; 4 = *Polybia rejecta*; 5 = *Polybia sericea*; 6 = *Polybia simillima*; 7 = *Synoeca septentrionalis*

D) Polistes, Ropalidini and Mischocyttarini wasps (reduced); 1 = *Belonogaser juncea colonialis*; 2 = *Mischocyttarus flavitarsus*; 3 = *Polistes canadensis*; 4 = *Polistes comanchus navajoe*; 5 = *Polistes dorsalis*; 6 = *Parachartergus fraternus*; 7 = *Polistes major castaneocolor*

E) Vespinae wasps (reduced); 1 = *Dolichovespula arenaria*; 2 = *Dolichovespula maculate;* 3 = *Vespula pensylvanica*; 4 = *Vespula vulgaris*; 5 = *Vespa luctuosa*; 6 = *Vespa simillima*; 7 = *Vespa tropica*

F) Solitary Wasps (reduced); 1 = *Dasymutilla chiron*; 2 = *Dasymutilla gloriosa;* 3 = *Scoliidae* ; 4 = *Stictia* sp.

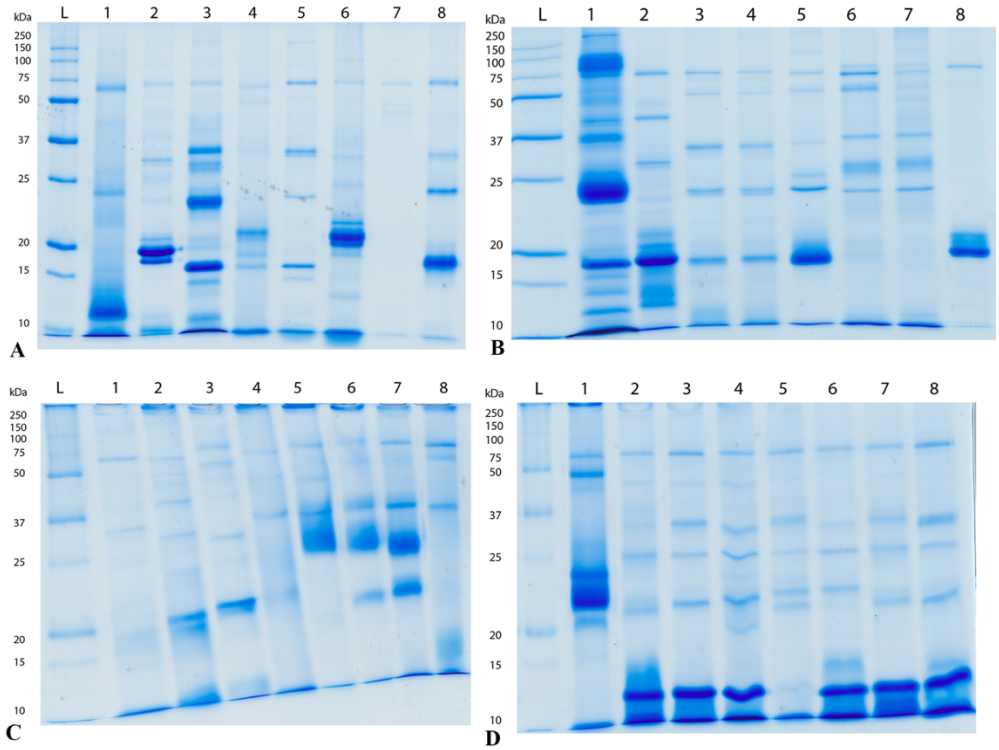

Figure 3.3. 1D SDS-PAGE Ants (reduced); A) 1 = *Paraponera clavata*; 2 = *Diacamma*; 3 = *Euponera sennaaren*; 4 = *Leptogenys*; 5 = *Neoponera villosa*; 6 = *Odontomachus*; 7 = *Opthalmopone;* 8 = *Megaponera analis*

B) 1 = *Pachycondyla crassinoda*; 2 = *Paltothyreus tarsatus*; 3 = *Platythyrea lamellosa*; 4 = *Platythyrea strigulosa*; 5 = *Streblognathus aethiopicus*; 6 = *Termitopone communtata*; 7 = *Termitopone communtata* (Queen); 8 = *Odontoponera*

C) 1 = *Ectatomma tuberculatum*; 2 = *Ectatomma*; 3 = *Gnaptogenys*; 4 = *Rhytidoponera metallica*; 5 = *Pogonomyrmex maricopa*; 6 = *Pogonomyrmex occidentalis*; 7 = *Pogonomyrmex rugosus*; 8 = *Diacamma*

D) 1 = *Tetraponera*; 2 = *Myrmecia browningii*; 3 = *Myrmecia gulosa*; 4 = *Myrmecia nigripes*; 5 = *Myrmecia pilosula*; 6 = *Myrmecia rufinodis*; 7 = *Myrmecia simillima*; 8 = *Myrmecia tarsata*

3.3.2. Proteomics

1D SDS-PAGE revealed the venoms of all social species showed clear homogeneity with only small variances in the peptides molecular masses (Figures 3.2. A, C – E, 3.3.). In contrast the venom profiles of the solitary species were noticeably more complex and diverse than their social counterparts (Figure 3.2. B, F). Despite the increased complexity of venom in the solitary species shown by the gels, significantly less proteins were identified by shotgun-MS/MS analysis (Table 3.3.), which is a likely artefact due to few homologous sequences available in the databases.

3.3.3. LC-MS

Venoms were also profiled using LC-MS. All venoms showed a similar generalised elution profile, that revealed venoms that were rich in low molecular weight components, this is supported by 1D gels and LC-MS/MS (Figures 3.4., 3.6., Table 3.3.). The components were distributed over the molar mass range of 500 – 14, 000 Da. However, even though no components with a higher molecular weight were identified in the chromatographs, this does not mean that these components were not present, rather that the known occurrence of ion suppression when using LC-MS may be hiding these other components [330]. The venoms of social bees showed similar chromatograms with evidence of some peptide variability between the species. However, the chromatograms of the venoms of the solitary bee species had distinctly less peaks, despite their proteomic profiles (Figure 3.2. B, Figure 3.4. F). The wasps all had significant similarities of retention times and molecular masses in their venom composition (Figure 3.5. A – D) as did the ants (Figure 3.6.). Solitary wasp venom chromatograms showed a diversity of molecular masses, with more peaks than were found in the social species (Figure 3.5. E, F). Further LC-MS chromatograms can be found in Appendix I (Figures S3.1. – 3.9.).

3.3.4. Functional assays

High presence of PLA_2 activity was found in all social bee venoms (Figure 3.7.), while the rest of Aculeata had comparatively lower levels. Statistical investigations provide support for social species being more likely to have higher PLA_2 activity (PGLS: $t = 3.27$, $df = 1$, $p = 0.002$). Contrastingly when looking at cleavage of matrix serine protease specific substrate, solitary bee venoms showed significantly higher activity than any other aculeate species (Figure 3.7.).

3.3.5. Cytotoxicity assay

The cytotoxic effects of crude venom on one healthy-type and one cancerous cell line were tested in order to ascertain generalised cytotoxicity (Figure 3.8.). The results showed that the majority of the social bees had strong cytotoxic tendencies on both cell lines, as did ants, in particular, the genus *Mymercia* (Appendix I Supplementary Tables 3.1., 3.2.). Using statistical measures, we found that increased cytotoxicity on both healthy and cancerous cell lines was related to social aculeates: MM96L (PGLS: $t = 3.22$, $df = 1$, $p = 0.002$); NFF (PGLS: $t = 2.87$, $df = 1$, $p = 0.005$). Further, increased cytotoxicity between the healthy and cancerous cell lines was also statistically significant (PGLS: $t = 10.92$, $df = 1$, $p = 2_e\text{-}16$).

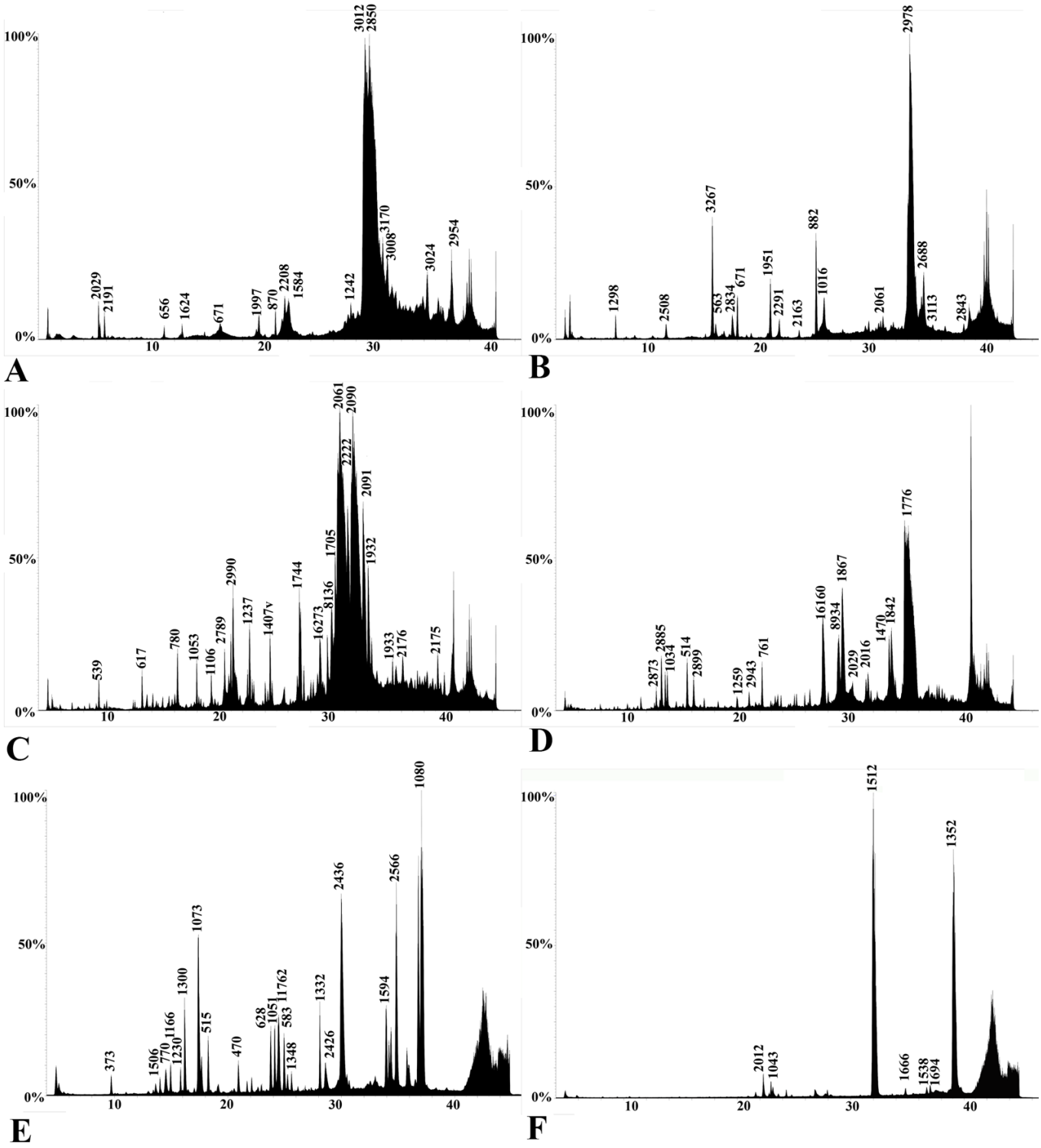

Figure 3.4. Representative LC-MS profiles of bee species A) *Apis mellifera* B) *Apis andreniformis* C) *Bombus impatiens* D) *Bombus sonorus* E) *Xylocopa californica* F) *Peponapis pruinosa* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

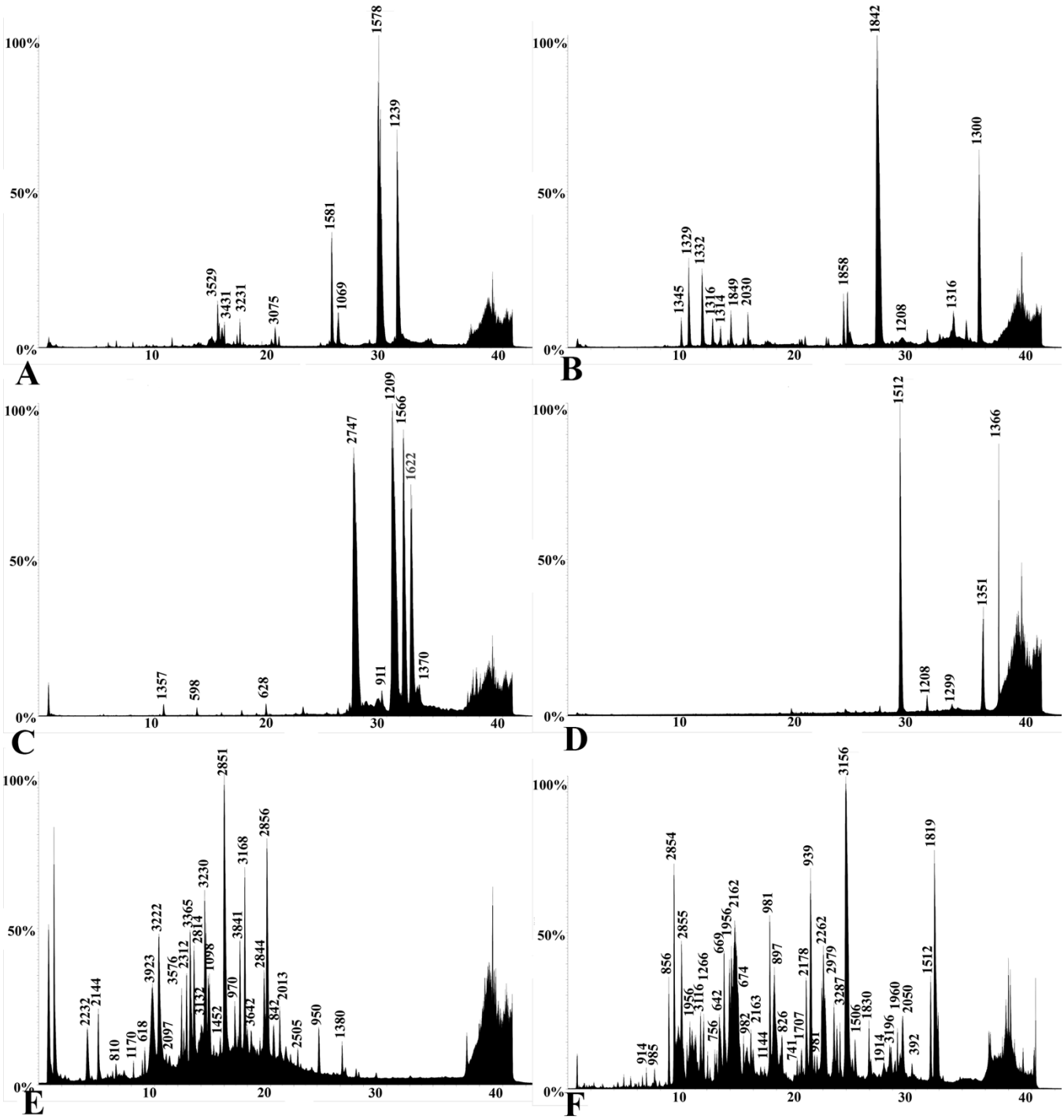

Figure 3.5. Representative LC-MS profiles of wasp species A) *Agelaia myrmecophila* B) *Polybia sericea* C) *Polistes major castaneocolor* D) *Vespula vulgaris* E) *Sticta* sp. F) *Dasymutilla klugii* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

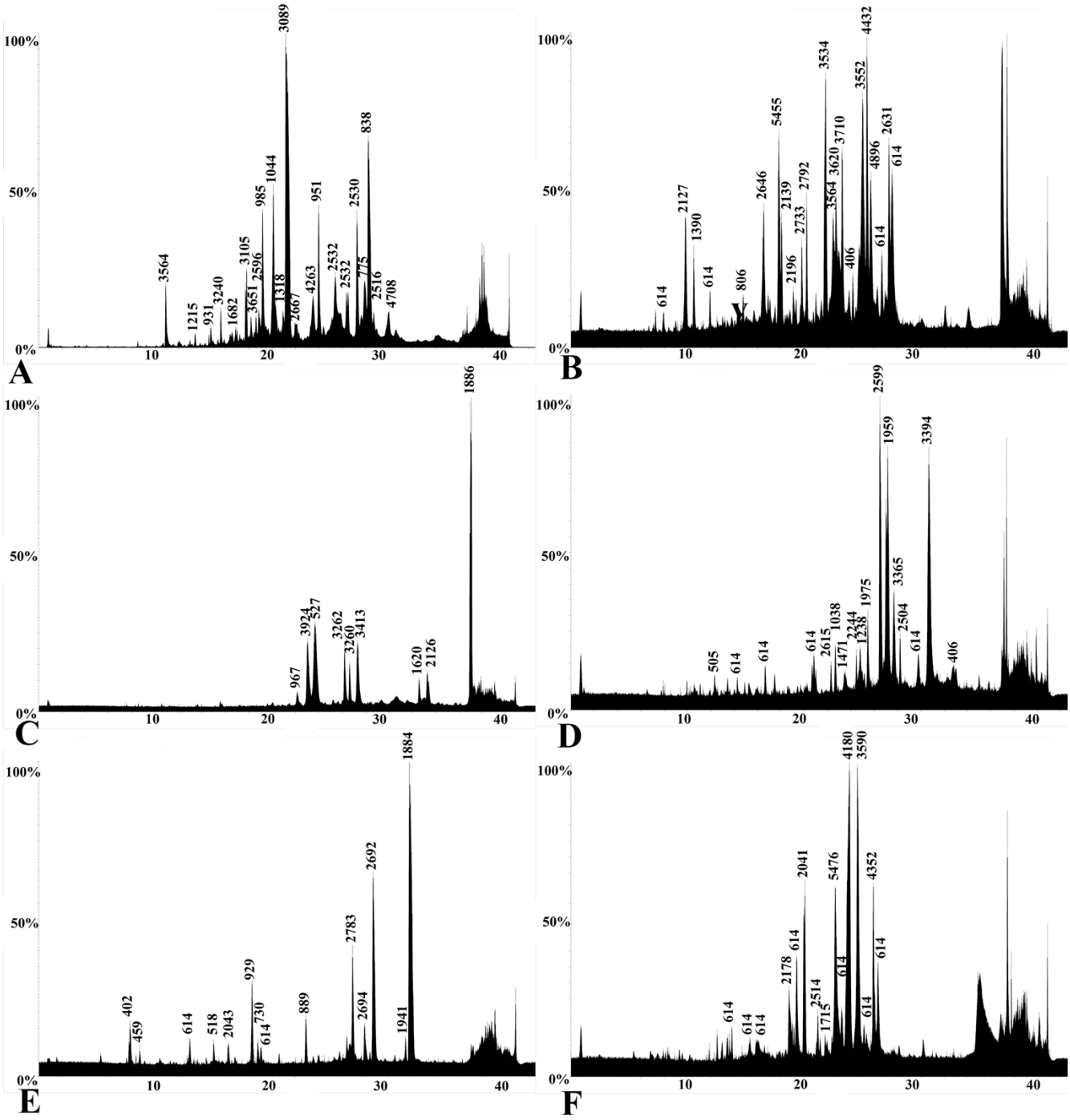

Figure 3.6. Representative LC-MS profiles of Formicidae species A) *Dinoponera gigantea* B) *Myrmecia rufinodis* C) *Pachycondyla crassinoda* D) *Platythyrea strigulosa* E) *Paltothyreus tarsatus* F) *Odontomachus* sp. X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

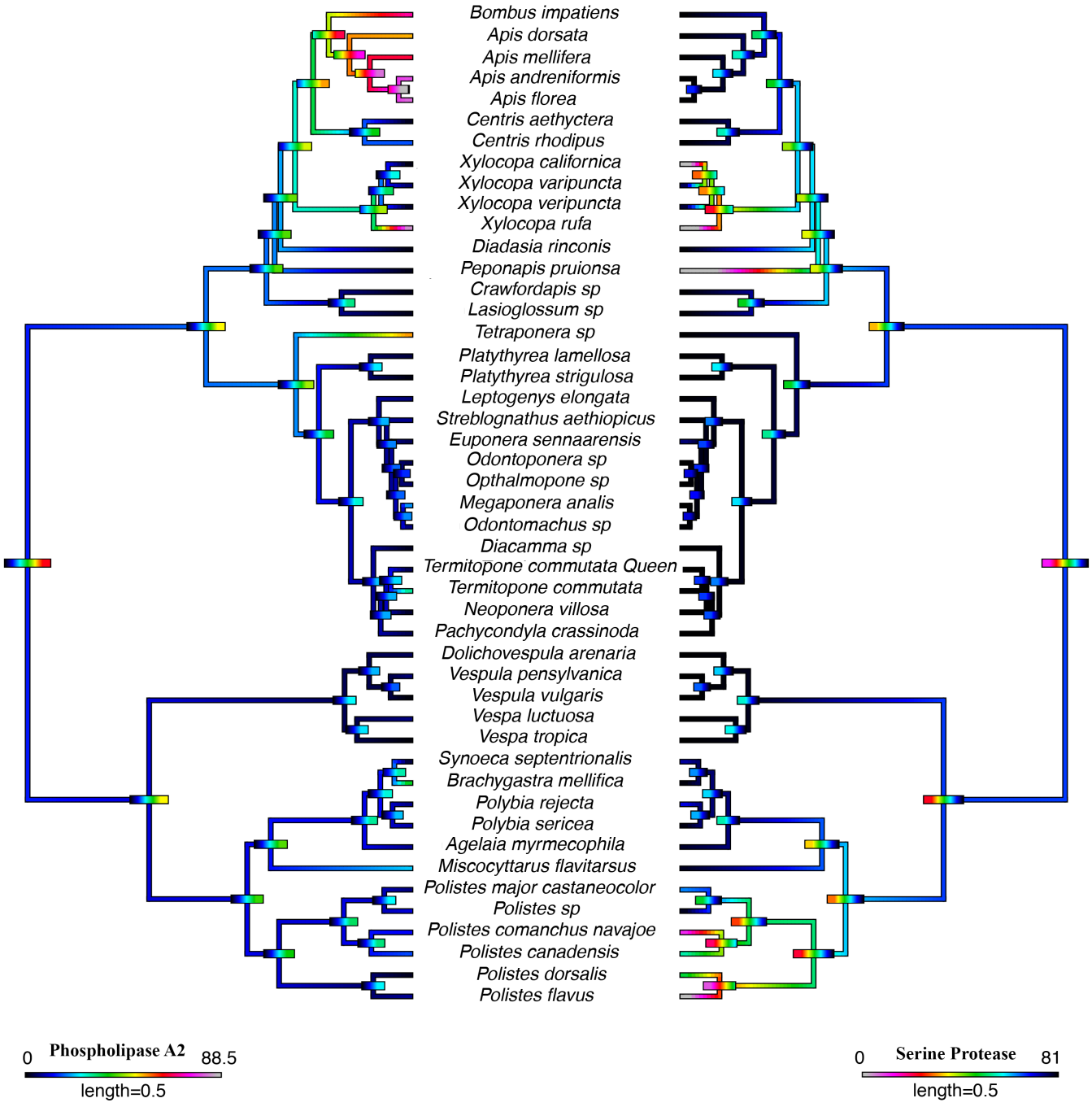

Figure 3.7. Ancestral state reconstructions of PLA_2 activity (left) and serine protease activity (right). Reconstruction over branches represents relative percentage absorbance where warmer colours represent higher activity.

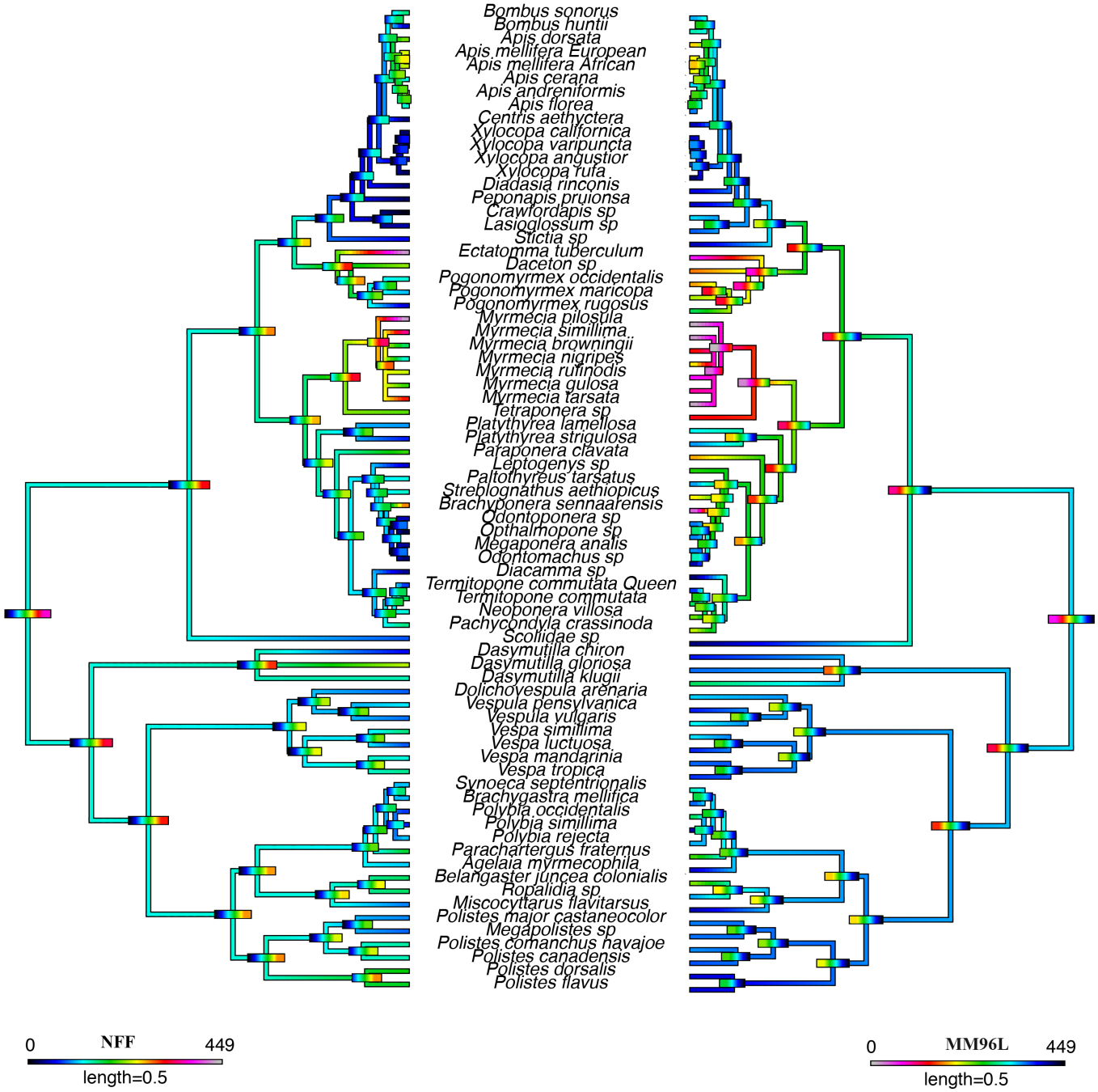

Figure 3.8. Ancestral state reconstructions of NFF cell line (left) and the melanoma (MM96L) cancer line. Reconstruction over branches represents AUC where warmer colours represent higher activity.

3.4. Discussion

In order to fully characterise the venom of aculeates, a comparative study of venom gland transcriptome and proteome is necessary. However, very few venom gland transcriptomes have been performed, with those that have being focused on social and parasitoid wasps and ants. This study includes the first venom gland transcriptome of any species of bee, as well as an extensive comparative overview of aculeate venoms as a group. Transcripts identified from the venom gland transcriptome of *A. mellifera* were found to have significant homology between the venom proteins of already sequenced toxins in the Uniprot database (Figure 3.1.). The absence of significant variation in the transcripts sequences highlights that honeybee venom, as a defensive venom, is not under the same selection pressures that predatory venoms are [47]. Instead, there was evidence of negative selection pressure against diversification, which is consistent with defensive venom being highly conserved in other lineages [47]. The finding of multiple transcripts that match various allergenic peptides is consistent with bee venoms being known to cause mainly inflammatory and immunological reactions in the victim [331] (Table 3.2.). Despite the identification of most of the major venom toxins, some of the previously described venom compounds were not able to be recovered. One of these was the antigen 5-like wasp venom paralog which was absent from the venom gland transcriptome. This venom protein is known to be seasonally expressed which may be the reason for its absence in this transcriptome [92].

Proteomic analysis combined with shotgun-MS/MS revealed a diversity of toxins present in both solitary and social species (Figure 3.2., 3.3.). The variability in the profiles of closely related species suggests potential presence of polymorphisms. Polymorphisms are instrumental in creating novel toxins with different selectivity and potencies. This increases the effectiveness of toxins across a broader range of predators [47]. The 1D SDS-PAGE results suggested that the solitary species of bees and wasps had more complex venoms than their social counterparts, with little conservation observed (Figure 3.2., 3.3.). This is consistent with the different ways their venoms are employed. Social aculeates primarily utilise their venom for defence and to protect their colonies. In contrast solitary bees are pollinivorous and rarely sting. Their venom secretions have been hypothesised to be used in order to protect the nest from bacterial or fungal infections rather than offense [75] and therefore are evolving in a putative chemical arms race with the microorganisms. Solitary wasps use their venom as a paralytic in order to immobilise and in some cases kill prey, meaning that they may also be prey-specific [50]. A negligible amount of toxins in solitary

species were identified using shotgun-MS/MS (Table 3.3.). This does not necessarily mean an absence of toxins but is most likely a result of the limited number of sequenced solitary venoms. However, this absence of results is disappointing as solitary wasp venoms are most likely rich in proteins that are used in order to kill and immobilise prey [50], while solitary bee venoms most likely are high in antimicrobial peptides [66, 67, 69, 73, 74, 131, 332]. Therefore, this aspect of the venom evolution unfortunately could not be considered in any depth in this project.

LC-MS results revealed a prevalence of low molecular weight molecules (Figures 3.4. – 3.6) that were consistent with previous studies suggesting high presence of biogenic amines in bees and wasps and alkaloids in ants [124, 146, 333] as well as allergens [57]. Allergens result in IgE-mediated reactions in humans and are most likely an important part in social species defensive strategy of social species [334]. Considering that social species attract large numbers of vertebrate predators and their venom are used primarily for defence [335], the presence of substantial quantities of peptides in the venom to generate an arsenal of toxins is especially important. Whereas the solitary species had a greater diversity of peaks, hinting at novel compounds not found in social aculeate venoms.

PLA_2 and serine protease are major allergens found in aculeate venoms. Here we assessed the activity of these enzymes across Aculeata. PLA_2 is known to be the main enzyme component found in honeybee venoms, making up approximately 12% of the dry weight of venom [335, 336]. Comparatively wasp venoms have been found to only have 0.1-1% of the protein present [70], ants have been found to have similarly low levels of PLA_2 [58]. The results of the social bee species in this study corroborate that social bees have higher levels of PLA_2 activity present in their venom, and that PLA_2 proteins are prevalent in aculeate venoms (Figure 3.7.).

As serine protease is another significant allergen found in aculeate venoms, the high levels of serine protease activity found in solitary bee species (Figure 3.7.) suggests that being stung by one of these species may induce a similar allergenic reaction that occurs when stung by a social species such as *A. mellifera* [57, 298, 337]. The molecular function of serine protease in bee venom is still unknown, however in arthropods the phenoloxidase enzyme (an innate immunity protein) is activated by serine protease [338, 339]. Its significant presence in solitary bee venom may indicate an importance in the immune response similar

to arthropods. Previous studies have shown that the immune response is reduced with the evolution of sociality, perhaps suggesting why the activity is much higher in some solitary species than social species [76].

Cytotoxicity in aculeate venoms has been well described [311, 315, 317, 340], however, whether it is a defensive adaptation or used for predatory purposes has not been shown. The results of this study suggest that cytotoxicity has evolved as a primarily defensive adaptation as there was found to be a significant relationship between social aculeates and strong cytotoxic effects on both healthy and melanoma cells (Figure 3.8.). The cytotoxic effects of the venom of *Apis* species is mainly contributed to the peptide melittin via a membranolytic effect [341], PLA_2 has also been shown to synergistically increase melittins cytotoxic effects [342]. Pilosulin isolated from the *Mymercia* genus has been identified as a potently cytotoxic molecule [317]. Social wasp venoms showed comparatively less cytotoxic activity. Cytotoxic molecules that have been identified in social wasps, include mastoparan, which targets the mitochondrial membrane resulting in mediatiating tumor cell cytotoxicity [343] and a biologically active quinone isolated from *Vespa simillima* venom which induces apoptosis [344]. Mastoparans have been isolated from solitary Vespidae but no other species of solitary wasps [99], perhaps hinting at their predominant role in causing the cytotoxic effects of these species. Solitary bee venom showed an absence of cytotoxic effects. As melittin is not present in solitary bee venom, this suggests that it could be one of the main drivers of cytotoxicity in social bee venom.

These differences in cytotoxicity between solitary and social venoms are most likely associated with the use of their venom and behaviour. Social venom is primarily used for defence and can also be used for predation. Solitary bee venom while also defensive is not used actively the way social bees use their venom, instead their venom is hypothesised to protect the nest from bacterial or fungal infections [75]. These results highlight the importance of cytotoxicity in defensive and predatory social aculeate venoms.

The use of venom peptides for cancer specific drugs is not a new area however, no molecule from venom has been approved for human use so far. This is mostly due to the difficulty in isolating peptides that are able to discriminate between deleterious cells and healthy cells. As found in this study aculeate venoms as whole entities are at best generalised cytotoxins and, therefore, have limited commercial use. Nonetheless studies have reported that

peptides from aculeate venoms have various anti-cancer and anti-tumour activities and thus are good potential candidates for these therapeutic avenues [310, 341, 345, 346].

3.5. Conclusion

This study aimed to address the paucity of information surrounding aculeate venoms as a whole and as such is the first large-scale analysis of aculeate venoms. It was also the first study that has sequenced the venom gland transcriptome of a honeybee, *A. mellifera*, showing that the toxins present are most likely under negative selection, fitting with their venom having a defensive function. Using a range of techniques, it was revealed that there are distinct differences between solitary and social with venoms in accordance with their lifestyles. Proteomics and mass spectrometry studies revealed the diversity of small peptides present in aculeate venoms. Whilst PLA_2 activity and cytotoxicity assays revealed significant differences between the venoms of social and solitary species, venom of social species showed a higher affinity for these activities. These components are mainly pain and/or damage inducing, suggesting that there is an increase of evolution pressure on the venom of social species in order to successfully ward off predators. While the venoms of social aculeates vary, their overall compositions, in terms of major toxins, allergens and prey reactions are remarkably similar, furthering underlining the similarities in their defensive behaviours. The demonstration of the diversity of toxins present in Aculeata is useful for researchers interested in isolating and characterizing novel toxins for use as investigational ligands or as scaffolds in drug design and development. To date this is the only known study to reveal the complexity of the venom composition across the diversity of aculeate species.

CHAPTER 4

Scratching the surface of an itch: Molecular evolution of Aculeata venom allergens

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Abstract

Hymenopteran insects are infamous for their sting, and their ability to cause severe anaphylaxis and in some cases death. This allergic reaction is a result of allergens present in the venom. Hymenopterans have many common venom allergens, the most widespread of which include phospholipase A1, phospholipase A2, acid phosphatase, hyaluronidase, serine protease and antigen 5. While there have been studies that look at the phylogenetic histories of allergens within closely related species, to our knowledge, this is the first study using evolutionary analyses to compare across Hymenoptera the types of selection that are occurring on allergens. This research examined the publicly available sequences of six different groups of allergens and found that allergens had diverged and formed closely related clades which share greater sequence similarities. We also analysed the patterns of selection and found that they are predominately under the influence of negative selection.

4.1. Introduction

Venoms are key evolutionary innovations that are found across a broad range of animal phyla [7]. Toxins recruited into venoms have been found to belong to only a handful of protein families and studying the evolutionary trajectories of these convergently recruited toxins will provide us with a better understanding of the mechanism of proteins and peptide neofunctionalisation. A major limitation of this type of research is the narrow taxonomical range studied, with entire groups being neglected. Hymenoptera, which represents one such neglected but highly speciose lineages of venomous animals, that have conquered virtually every terrestrial ecosystem [1]. Their venom is constituted by a mixture of proteins, peptides and low molecular weight compounds, and is employed for antipredator defence of the individual and/or the entire colony, as well as for prey capture [335]. Multiple proteins present in hymenopteran venoms are allergenic and are most commonly associated with local and systemic allergic reactions. As a result, stings caused by hymenopterans are one of the main causes of IgE-mediated anaphylaxis among the human population [347, 348].

Allergens play an important role in the defence of hymenopteran insects. Schmidt [334] proposed that these toxins confer an evolutionary advantage as they induce learned avoidance in predators. Despite their tremendous ecological importance, there is a lack of understanding of the evolutionary origin and diversification of venom allergens across hymenopteran species. Because of their relatively deep origin (280 MYA), hymenopterans are an ideal system to investigate the dynamics of venom across a long evolutionary period [22]. Further, understanding the molecular evolution of allergens may help in determining

their roles in hymenopteran venoms, as well as facilitating improvements in the therapeutic treatments for allergic reactions and hypersensitivity to stings.

In this study, we examine phylogenetic histories and the molecular evolution of the major venom allergens and provide the first comprehensive overview of Hymenoptera venoms. This study examines the evolution of major venom allergens in hymenopteran venoms, including phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), hyaluronidase, acid phosphatase, serine protease and antigen 5 (ag5).

4.2. Materials and methods

4.2.1. Phylogenetic Reconstruction

These six allergens were selected as they are the major venom allergens identified in hymenopteran venoms [57, 349]. Protein sequences for each hymenopteran allergen were pulled from the UniProt database. The sequences were aligned using a combination of manual alignment of the conserved cysteine positions and alignment using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm implemented in AliView for the blocks of sequence in between these sites [350, 351]. We reconstructed the phylogeny of these sequences using MrBayes 3.2 for 15,000,000 generations and 1,000,000 generations of burnin with lset rates=invgamma (allows rate to vary with some sites invariant and other drawn from a γ distribution) and prset aamodelpr=mixed (allows MrBayes to generate an appropriate amino acid substitution model by sampling from 10 predefined models) [352]. The run was stopped when convergence values stabilized at approximate 0.013.

4.2.2. Tests for Selection

Coding DNA sequences were compiled from GenBank [353]. The sequences were trimmed to only include those codons, which translate to the mature protein, translated, aligned, and reverse translated using AliView and the MUSCLE algorithm [350, 351]. Phylogenetic trees for each clade were generated from the resulting codon alignments using the same methods as described above. This tree topology was used for all subsequent analyses. We used several of the tests for selection implemented in HyPhy version 2.220150316beta due to their different emphases [354]. The AnalyzeCodonData analysis generates overall ω values for an alignment while the FUBAR method gauges the strength of consistent positive or negative selection on individual amino acids [355]. In contrast, the MEME method identifies individual sites that were subject to episodes of positive selection in the past [356].

4.2.3. Protein Modelling

Custom models for each clade were generated by inputting representative sequences to the Phyre2 webserver using the Intensive option [357]. Alignments of each clade were trimmed to match these structures and attribute files were created from FUBAR and MEME results. The structures were rendered and coloured according to these attributes in UCSF Chimera version 1.10.2 [358].

4.3. Results

We investigated the nature of natural selection influencing the evolution of genes encoding various hymenopteran allergens by computing the ratio of non-synonymous (dN) to synonymous (dS) substitutions, called omega (ω), where ω less than, greater than or equal to one is characteristic of negative, positive and neutral selection, respectively. Fast Unconstrained Bayesian AppRoximation (FUBAR) and Mixed Effects Model of Evolution (MEME) were also employed. FUBAR detects sites evolving via pervasive diversifying and purifying selection and MEME identifies sites under episodic diversifying selection.

4.3.1. Phospholipases

Our phylogenetic analysis showed that hymenopteran PLA_1 belongs to two distinct monophyletic clades (counting those with at least five sequences) (monophyletic in this instance meaning groups within this selection of sequences that form clades) (Figure 4.1.). Clade A consisted of Formicidae species and clade B of Vespidae species. Since all Formicidae sequences are more closely related to one another than to Vespidae sequences and vice versa, it appears that the diversification of this toxin family occurred independently in both families after their divergence. Although purifying selection largely influences both clades, clade B has a lower overall ω value than clade A (ω = 0.36 and 0.56 respectively) and has far more sites that were found to be significantly under purifying selection (110 and 16 sites respectively) and fewer sites under diversifying selection (1 and 5 sites respectively) according to FUBAR (Table 4.1.). MEME, however, identified more sites that have been subject to episodic diversifying selection in clade B (28 sites) than clade A (10 sites; Table 4.1.). Figure 4.2. applies the values generated by these site-specific analyses to protein structures predicted by the Phyre2 server, which shows that despite the lower overall ω value, clade B possesses several specific residues that are subject to diversifying selection.

Phylogenetic analysis of hymenopteran PLA_2 shows that these proteins belong to at least 3 distinct monophyletic clades; two distinct groups of Apidae species and one group of Formicidae species (Figure 4.3.). The rates and patterns of molecular evolution in the clades are similarly under the influence of purifying selection, with low ω values ranging between 0.12 – 0.34 (Table 4.1.). FUBAR method identified between 35 – 76 negatively selected sites and MEME identified between 2 – 9 sites under episodic selection. Protein modelling showed that the majority of sites under positive and episodic selection are on the surface of the protein structure (Figure 4.4.).

Table 4.1. Tests of selection on the Hymenoptera allergens

Phospholipase A_1					
Clade	ω	FUBAR (-)	FUBAR (+)	MEME	FUBAR & MEME
A	0.56	16	5	10	0
B	0.36	110	1	28	1
Phospholipase A_2					
Clade	ω	FUBAR (-)	FUBAR (+)	MEME	FUBAR & MEME
A	0.40	28	3	12	3
B	0.37	8	2	1	0
C	0.13	81	2	2	0
Acid Phosphatase					
Clade	ω	FUBAR (-)	FUBAR (+)	MEME	FUBAR & MEME
A	0.18	197	0	21	0
B	0.18	230	2	16	2
C	0.06	216	0	4	0
D	0.28	189	4	35	0
Hyaluronidase					
Clade	ω	FUBAR (-)	FUBAR (+)	MEME	FUBAR & MEME
A	0.15	249	0	25	8
B	0.21	22	0	3	0
Serine Protease					
Clade	ω	FUBAR (-)	FUBAR (+)	MEME	FUBAR & MEME
A	0.11	191	0	7	0
B	0.27	231	2	21	1
C	0.31	191	6	32	4
D	0.55	104	10	54	6
E	0.16	213	0	8	0
Antigen 5					
Clade	ω	FUBAR (-)	FUBAR (+)	MEME	FUBAR & MEME
A	0.85	0	0	0	0

B	0.73	4	0	66	0
C	0.95	1	1	59	1
D	0.85	1	1	13	0
E	1.06	0	0	0	0
F	0.78	0	1	0	0
G	0.66	0	0	3	0

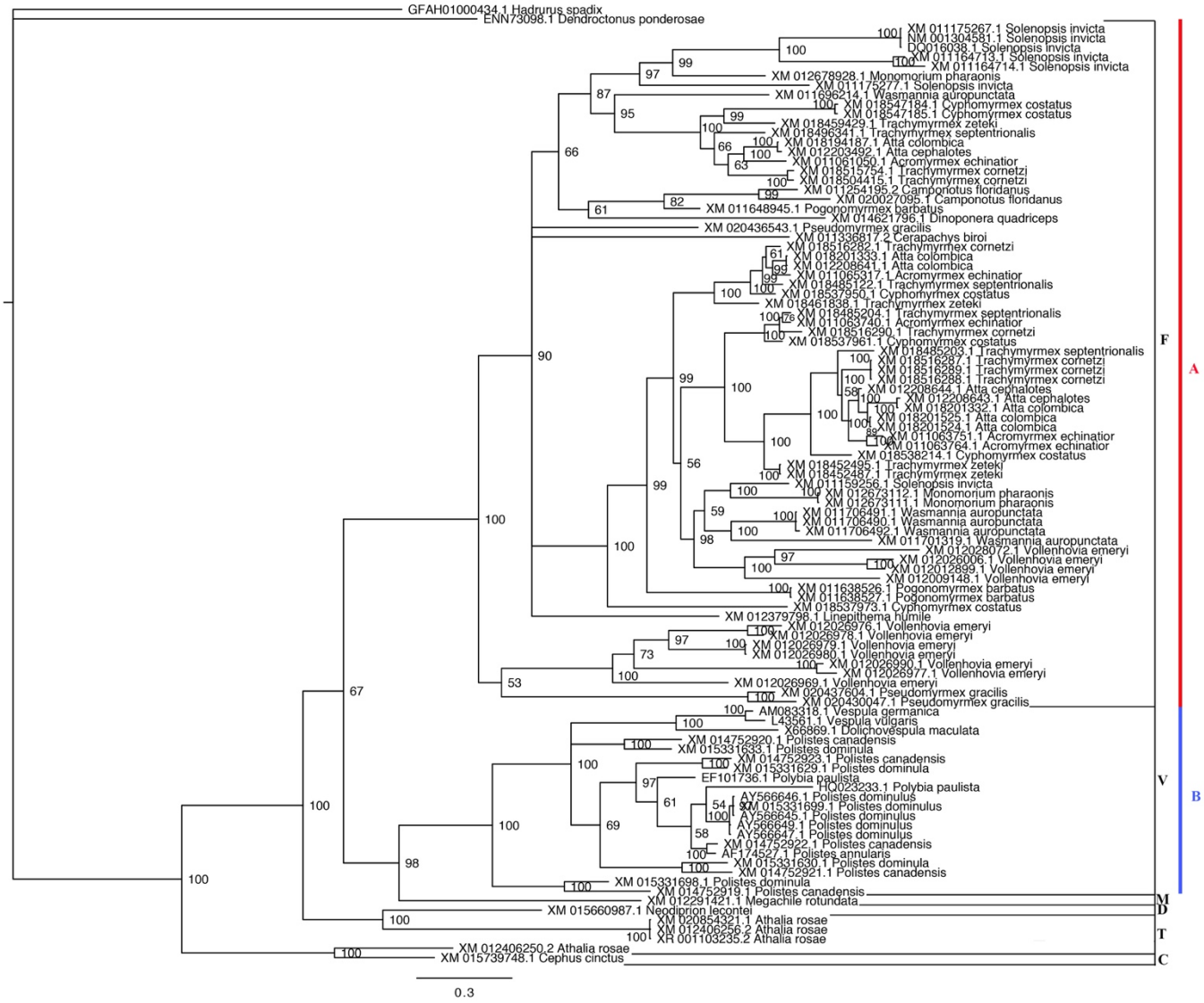

Figure 4.1. Phylogenetic tree of publicly available hymenopteran phospholipase A_1 sequences. Where A and B represent closely related groups. Scale bar represents an average of 0.3 substitutions per site

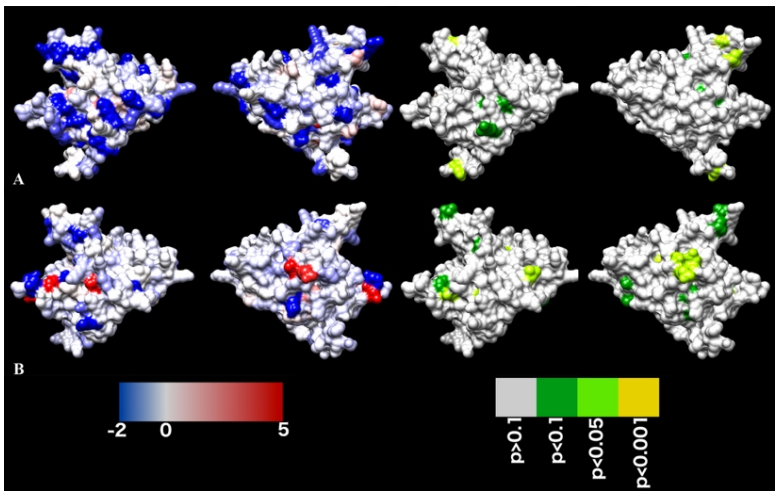

Figure 4.2. Protein models of phospholipase A_1. Show front and back views coloured according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

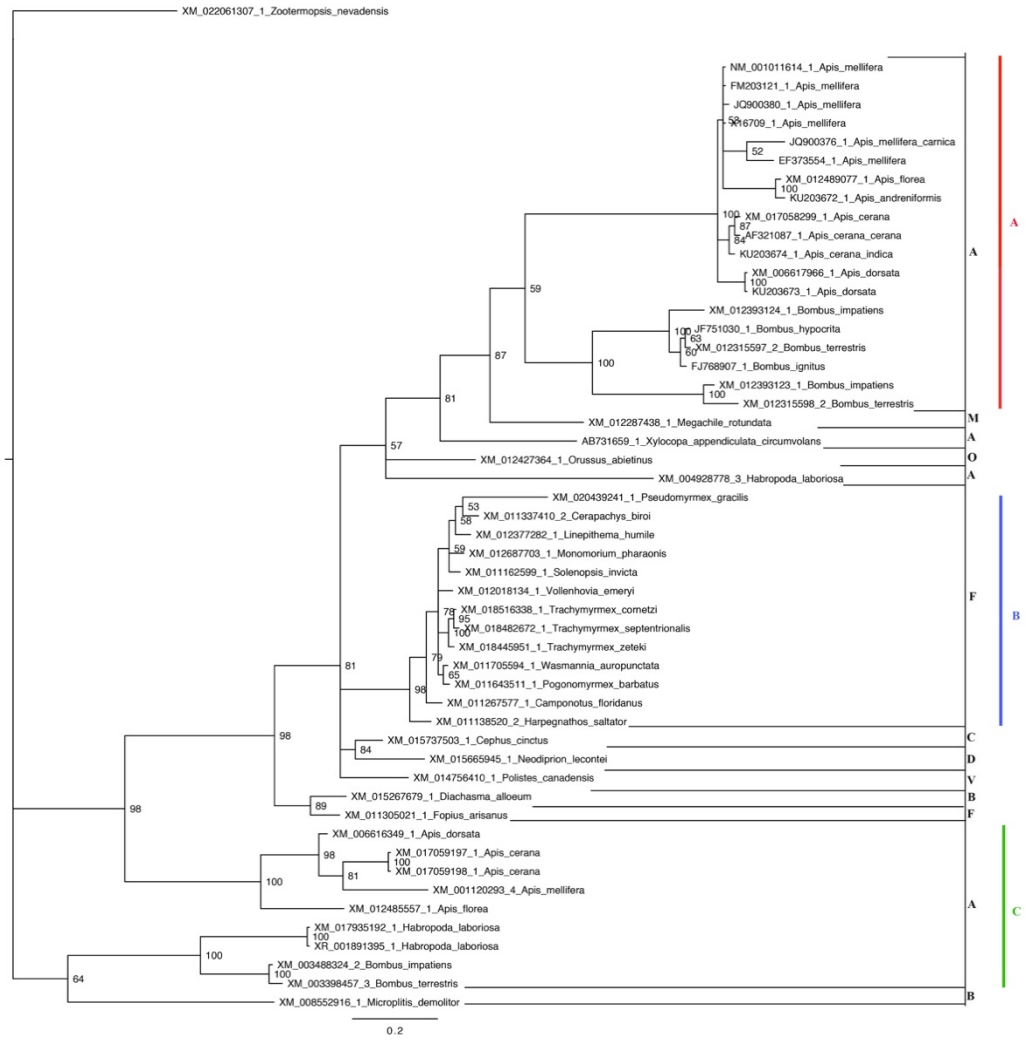

Figure 4.3. Phylogenetic tree of publicly available hymenopteran phospholipase A_2 sequences. Where A, B and C represent closely related groups. Scale bar represents an average of 0.2 substitutions per site

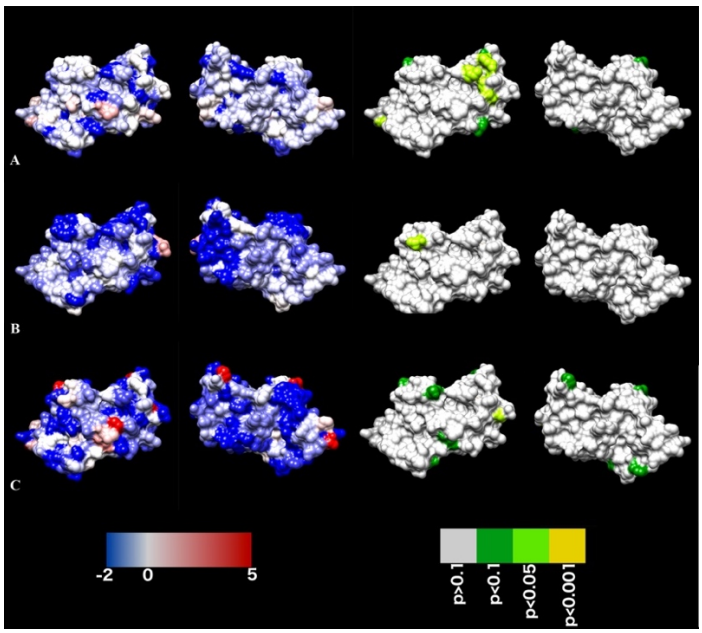

Figure 4.4. Protein models of phospholipase A_2 Show front and back views coloured according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

4.3.2. Acid phosphatase

Phylogenetic analysis of the hymenopteran acid phosphatase enzyme showed four distinct monophyletic clades. Clade A and D were formed solely by Formicidae species, while Clade B was comprised of Apidae and Clade C encompassed a variety of parasitoid wasps and sawflies. The rates and patterns of evolution in the clades of acid phosphatase had many similarities, all influenced by purifying selection. There were consistent low ω values (0.06 – 0.28), FUBAR method identified numerous sites (189 – 230) under negative selection, while MEME identified only a small number of sites (4 – 35) as having experienced episodes of diversifying selection (Table 4.1.). Figure 4.6. combines these tests for selection with protein structures predicted by the Phyre2 server, showing the extent to which these genes are dominated by purifying selection.

4.3.3. Hyaluronidase

Phylogenetic analysis of the hymenopteran hyaluronidase enzyme shows that they belong to two distinct monophyletic clades (Figure 4.7.). Clade A consists of Braconidae parasitoid wasps and clade B is comprised of Vespidae species. Both clades show similarly low ω values 0.15 and 0.21 respectively (Table 4.1.). Despite this, FUBAR identifies many more negatively selected sites in clade A and MEME also identifies more sites under the influence of episodic diversifying selection in clade A. Figure 8 uses both FUBAR and MEME tests for selection with predicted protein structures to provide further phylogenetic context.

4.3.4. Serine Protease

Our phylogenetic analysis showed that hymenopteran serine protease enzyme belongs to five distinct monophyletic clades (Figure 4.9.). These clades show that the serine protease sequences were quite diverse. Clade A is comprised of parasitic wasps and sawfly's, clade B Apoidean bees, clades C and D consist of all of the Formicidae species and clade E the Braconidae wasps. There were minimal differences in the rates and patterns of molecular evolution between the clades (Table 4.1.). All clades had a low ω values (0.11 – 0.55), and significant number of sites that FUBAR identified as being negatively selected (204 – 231). MEME identified as many as 54 sites evolving under the influence of episodic diversifying selection in clade D and as little as 7 sites in clade A. On all of these measures, Clades A and E exhibited stronger purifying selection. Figure 4.10. combined the FUBAR and MEME tests for selection with protein structures predicted by the Phyre2 server in order to provide additional phylogenetic context.

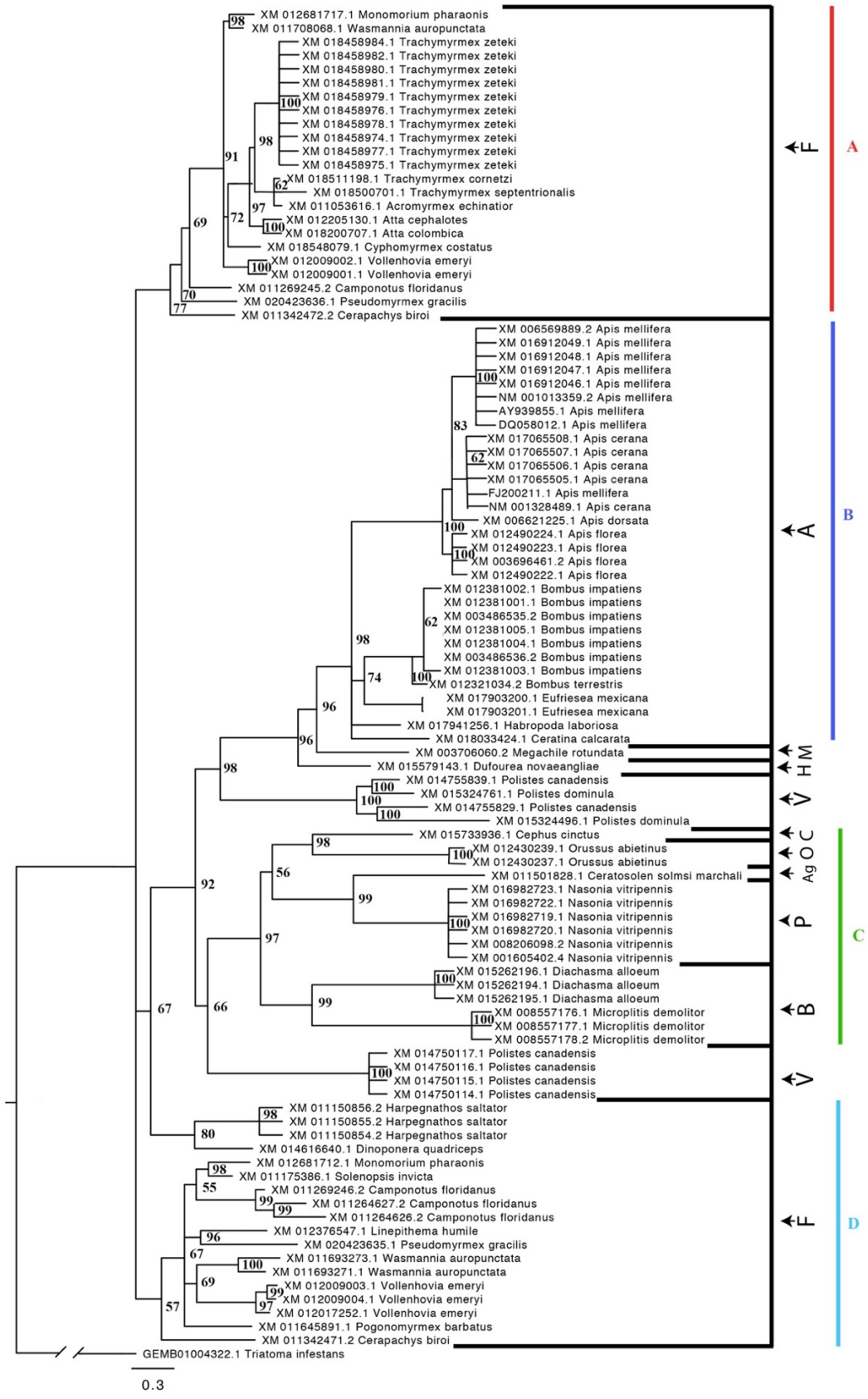

Figure 4.5. Phylogenetic tree of publicly available hymenopteran acid phosphatase sequences. Where A, B, C and D represent closely related groups. Scale bar represents an average of 0.3 substitutions per site

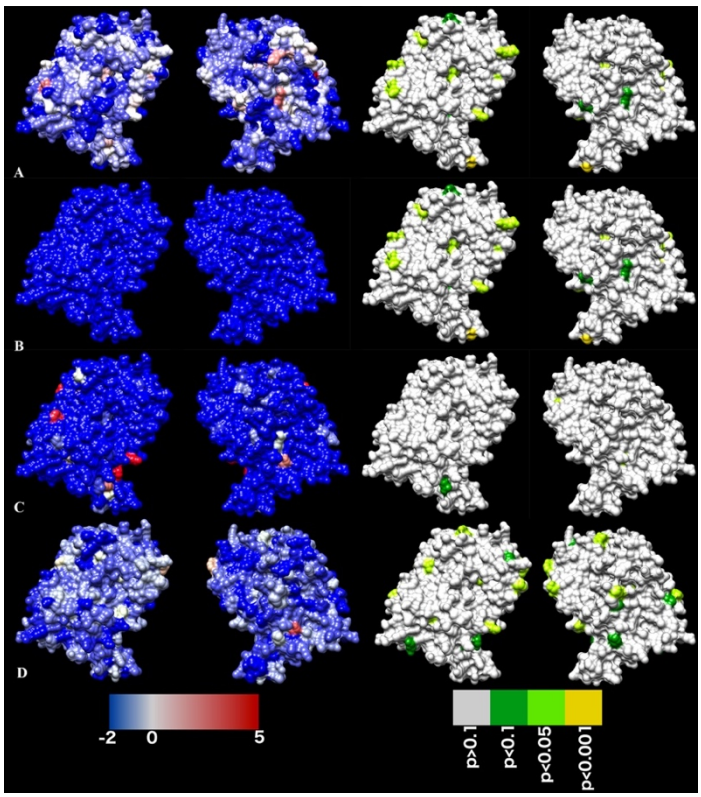

Figure 4.6. Protein models of acid phosphatase. Show front and back views coloured according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

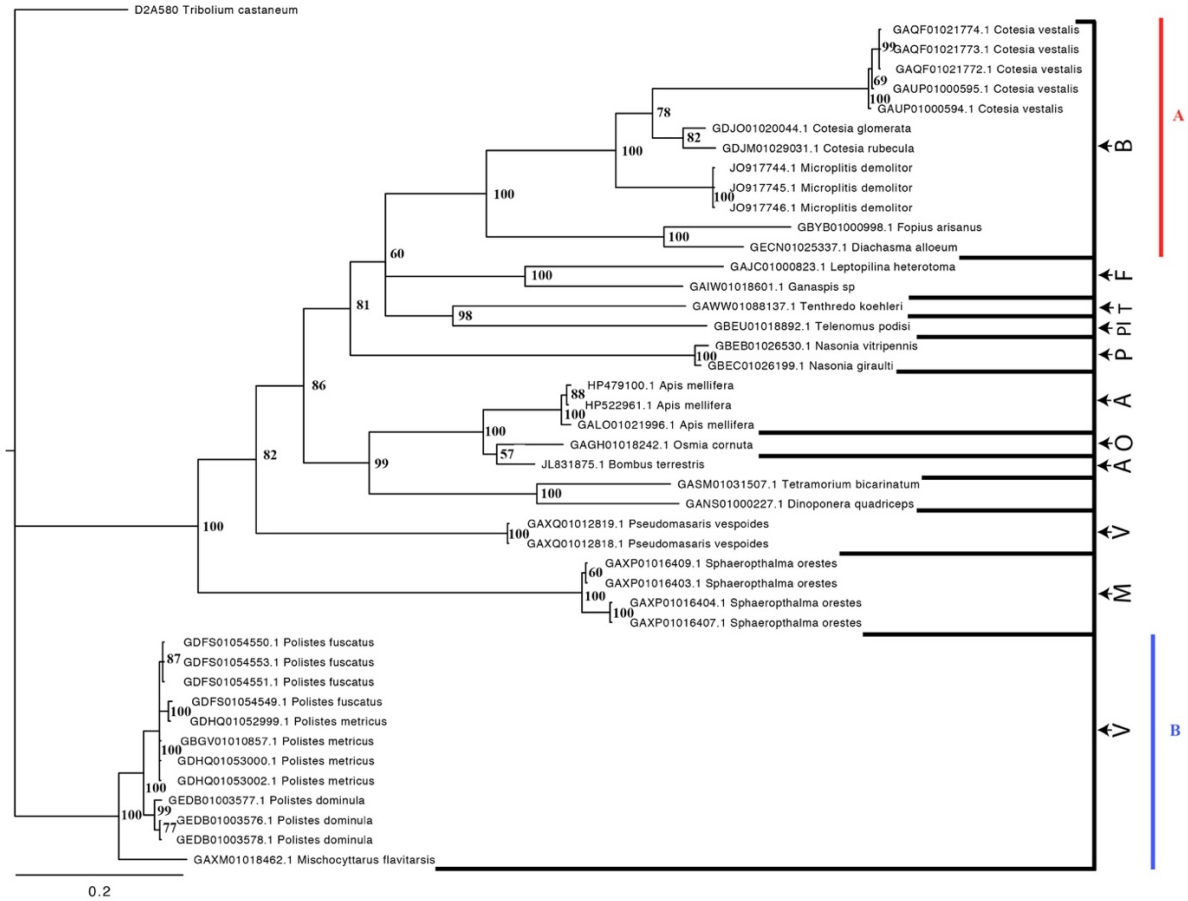

Figure 4.7. Phylogenetic tree of publicly available hymenopteran hyaluronidase sequences. Where A and B represent closely related groups. Scale bar represents an average of 0.2 substitutions per site

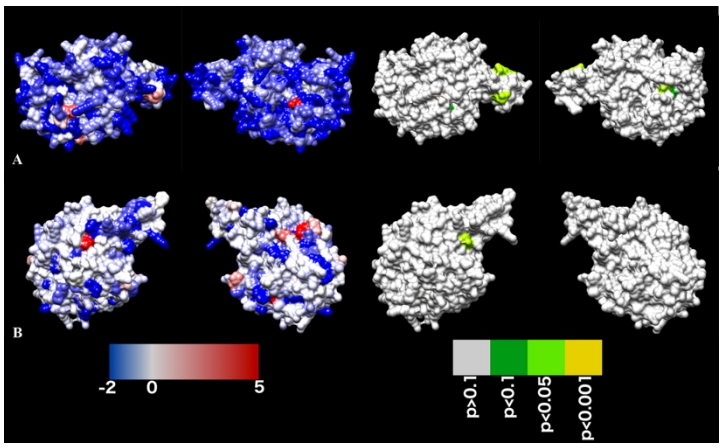

Figure 4.8. Protein models of hyaluronidase. Show front and back views coloured according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

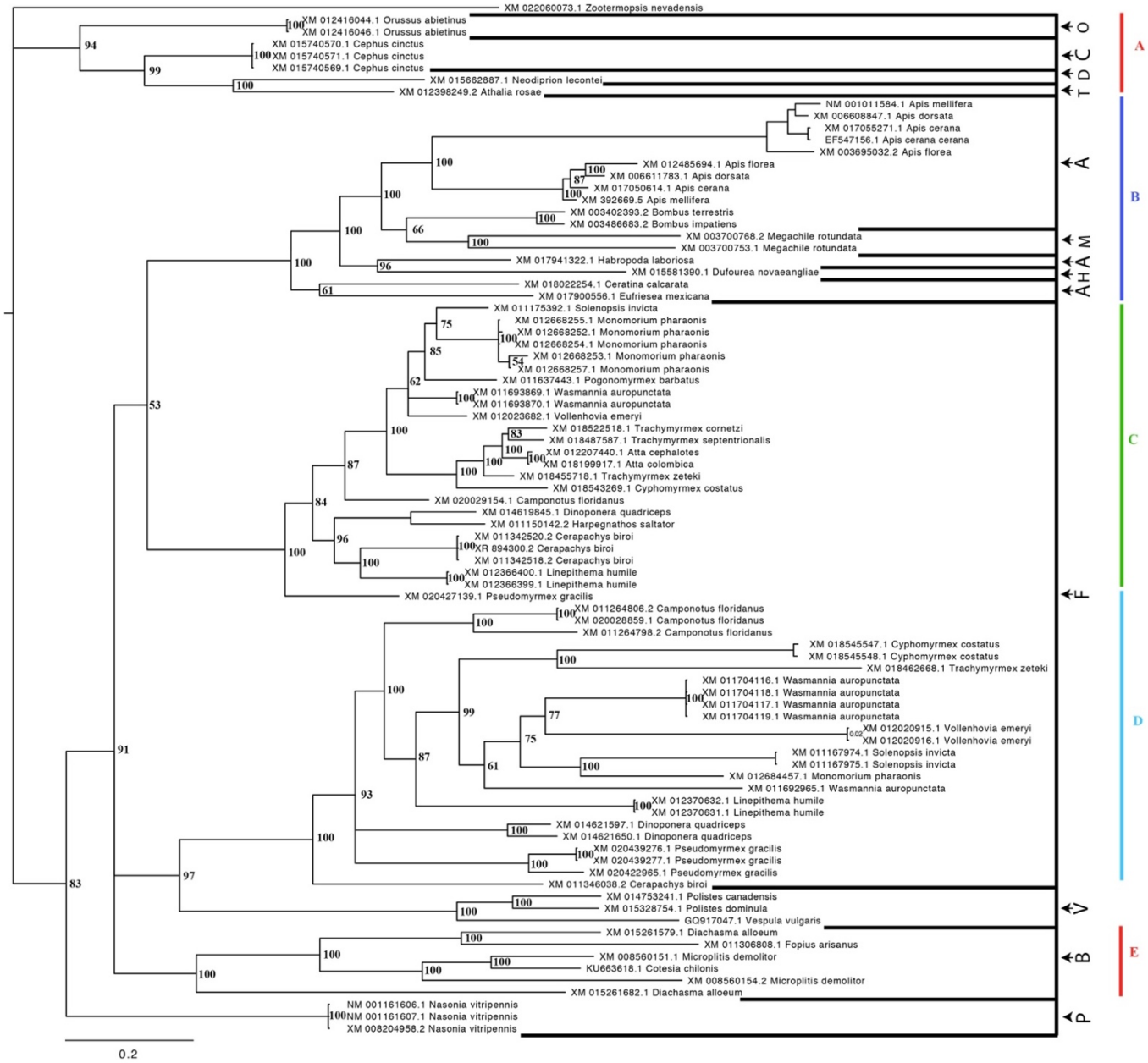

Figure 4.9. Phylogenetic tree of publicly available hymenopteran serine protease sequences. Where A, B, C, D and E represent closely related groups. Scale bar represents an average of 0.2 substitutions per site

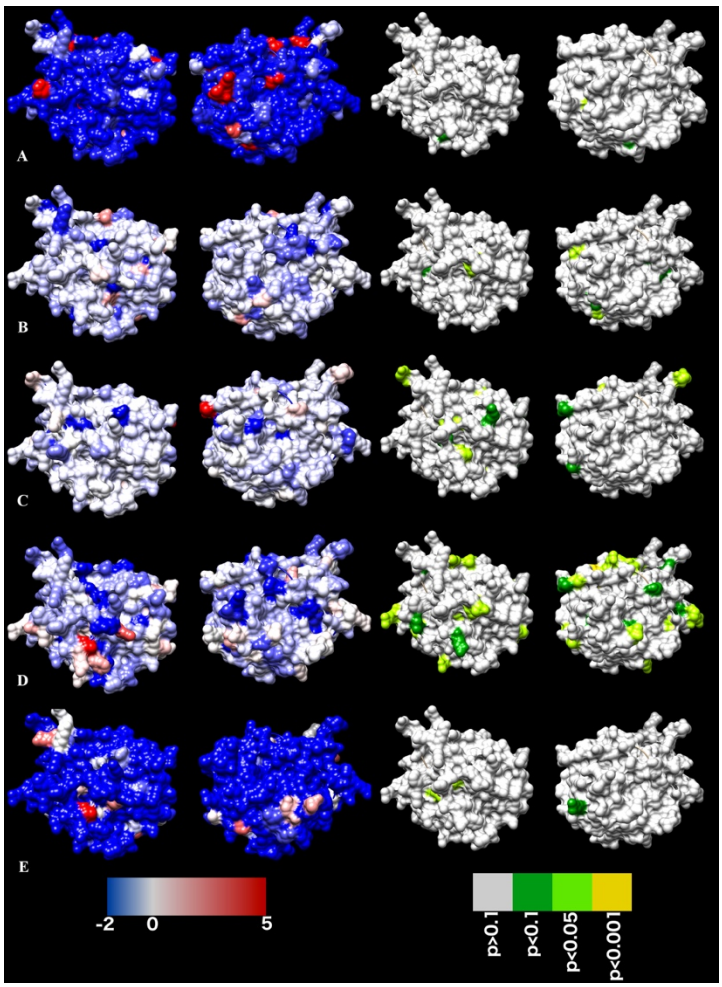

Figure 4.10. Protein models of serine protease. Show front and back views coloured according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

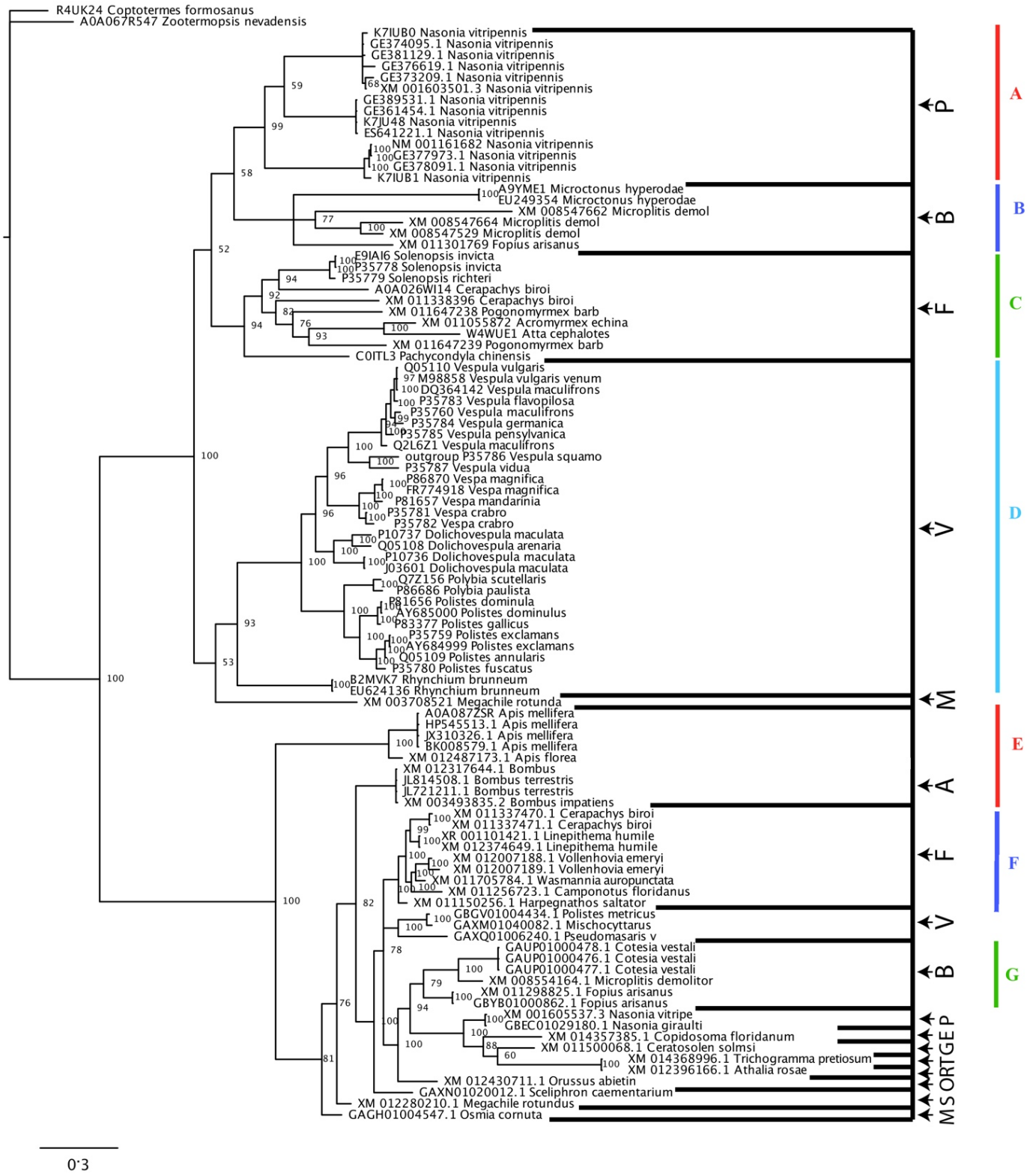

Figure 4.11. Phylogenetic tree of publicly available hymenopteran antigen 5 sequences. Where A, B, C, D, E, F and G represent closely related groups. Scale bar represents an average of 0.2 substitutions per site

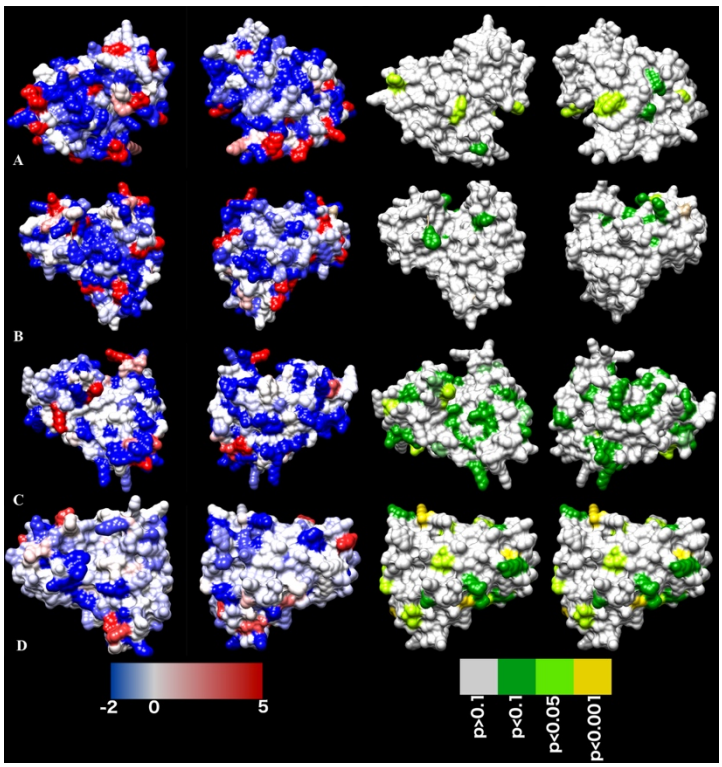

Figure 4.12. Protein models of antigen 5. Show front and back views coloured according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

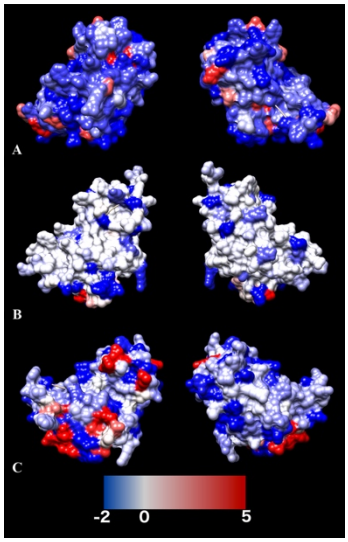

Figure 4.13. Protein models of antigen 5-like. Show front and back views coloured according to FUBAR's estimated strength of selection (β-α)

Table 4.2. Known functional activities of Allergens

Allergens	Known Activity in Hymenoptera venom	Known activity in other venoms	References
PLA_2	Hydrolase	Hydrolase, Myotoxin, Neurotoxin, Presynaptic neurotoxin	[359, 360]
PLA_1	Hydrolase	-	[360]
Acid phosphatase	Hydrolase	Hydrolase	[360]
Hyaluronidase	Hydrolase, Glycosidase	Hydrolase, Glycosidase, Antiedematogenic activity	[360, 361]
Serine protease	Hydrolase, Blood coagulation cascade activating toxin, Fibrinolytic toxin, Hemostasis impairing toxin, Protease, Prothrombin activator	Hydrolase, Hemostasis impairing toxin, Platelet aggregation activating toxin, Fibrinogenolytic toxin, Blood coagulation cascade inhibiting toxin, Calcium channel impairing toxin, Calcium-activated potassium channel impairing toxin, Ion channel impairing toxin, Neurotoxin, Potassium channel impairing toxin, Protease inhibitor, Serine protease inhibitor, Voltage-gated calcium channel impairing toxin	[360, 362, 363]
Antigen 5	-	Calcium channel impairing toxin, Calcium-activated potassium channel impairing toxin, Ion channel impairing toxin, Potassium channel impairing toxin, Ryanodine-sensitive calcium-release channel impairing toxin, Voltage-gated potassium channel impairing toxin, Neurotoxin	[360, 364, 365]

4.3.5. Antigen 5

Our phylogenetic analyses showed that hymenopteran ag5 and ag5-like sequences phylogenetically distinct (Figure 4.11.). The ag5 sequences make up four clades consisting of Pteromalidae, Braconidae, Formicidae and Vespidae. The ag5-like sequences fall into 3 clades; Apidae, Formicidae and Braconidae.

There were clear differences in the rates and patterns of molecular evolution between the clades (Table 4.1.). Ag5 were under weak negative selection with ω values ranging from 0.58 – 0.95. There were very few sites under negative or positive selection as identified by FUBAR and MEME. However, as many 66 sites were identified to be under episodic diversifying selection. In contrast ag5-like sequences while also under weak purifying selection (0.66, 0.78) and one clade, E was under weak positive selection (1.06). No sites

were under negative selection as identified by FUBAR and only clade G had any sites under episodic diversifying selection. Figures 4.12. and 4.13. display the FUBAR and MEME results with protein structures predicted by the Phyre2 server. It shows that most of the residues under diversifying selection are highly exposed on the surfaces of the proteins.

4.4. Discussion

4.4.1. Strong negative selection influences the evolution hymenopteran allergens

Molecular evolutionary assessments of hymenopteran allergens show that all with the exception of ag5 and ag5-like toxins are subject to extreme evolutionary conservation (Table 4.1.). The ω values ranged between 0.06 – 0.56, indicating a strong influence of negative selection on a majority of sites in these proteins. This fits with the previous findings that, overall, the venoms of ancient lineages evolve under heavy constraints of negative selection, while venoms in relatively recent lineages are more likely to be evolving under the influence of positive selection, such as snake metalloprotease and three-finger toxins [9, 11-17].

The use of FUBAR identified a number of sites (1- 10) under positive selection while MEME identified several sites (ranging 3 to 54) across the allergens that experienced episodic bursts of adaptive selection (Table 4.1.). Similar phenomenon have been found in scorpions [23] and cnidarians [19], both of which are ancient venomous lineages originating ~400 [366] and ~600 million years ago (mya) [367], respectively. These studies suggest that variation in venom-encoding genes accumulate episodically, likely under evolutionary pressure from prey and predators or shifts in ecology [20]. When toxins that are beneficial originate they become fixed in the population and undergo purifying selection. Hymenoptera began to diversify ~281 mya [22], suggesting that they may follow the same pattern of purifying evolution. This is in contrast to advanced snakes and cone snails, which are comparatively evolutionary younger lineages and show a more pronounced rapid evolution of genes under positive selection [13, 15].

4.4.2. Sites under episodic selection are surface accessible

Venom proteins involved in predation have been suggested to evolve through rapid accumulation of variation in the exposed residues (RAVER). This is where the surface of the toxin accumulates the bulk of variations under positive selection while the core residues involved in stability and activity are conserved [15]. Mutations leading to the loss of stable structure and function are removed by purifying selection, and structurally and catalytically

important residues are conserved. Additionally, accumulations of variations on the surface of a protein are advantageous to altered surface chemistry potentially leading to neofunctionalisation.

Evolution through RAVER is congruent with the 3D models of the allergen structures where the majority of positively selected or episodically adaptive sites are surface exposed (Figures 4.7. – 4.14.). RAVER has been established in multiple venom linages, and it appears that even allergens from hymenoptera adopt RAVER and favour accumulation of variation on the molecular surface [11, 15, 16, 368, 369]. Certain codon sites are under the influence episodic diversifying selection; they are mostly concentrated to the surface of the allergen, likely a consequence of adaption to host immune responses. This favouring of episodic evolution on the surface of the protein may be one of the reasons that observed clinical cross-reactivity between families is low [370].

Despite some codons occurring on the surface of the allergen presenting under positive selection, the majority of codons were still under negative selection. These conserved codons may be the site of IgE-binding epitopes. Tree pollen allergens have structurally conserved molecular surfaces that are the basis for allergic cross-reactivity. Mirza et al. [371] suggested that these conserved codons on the molecular surface of the allergen harbour major IgE-binding epitopes. This may also be the case for hymenopteran allergens; however, further characterisation of the molecular surface is required in order to determine this.

4.4.3. Antigen 5 under neutral selection

Our phylogenetic analyses show that hymenopteran ag5s have a complex evolutionary history with frequent gene duplications and losses. The ag5-like proteins found in *Apidae* venoms while clearly related to the group of ant and wasp venom ag5 proteins, form their own distinct clade (Figure 4.11.). Ag5-like proteins were also identified in multiple other Hymenoptera species. The ω values for ag5 proteins ranged between 0.73 – 0.95, indicating a weak influence of negative selection on a majority of sites in these proteins (Table 4.1.). Ag5-like show similar ω values, with the exception of clade E comprised of Apidae species, at 1.06 is under weak positive selection. The ω and FUBAR values suggest that the protein is under neutral selection, while MEME values indicate in ag5 proteins there are multiple sites under going episodic diversifying selection (0 – 59 sites).

4.4.4. Allergen functionality

Allergens have been shown to belong to a small number of protein families that present with limited molecular functions [372]. Of the known allergens one-sixth have hydrolase activity. Hydrolase activities are present in hymenopteran allergens such as phospholipases, acid phosphatase, hyaluronidase and serine protease (Table 4.2.). However, functional activity of hymenopteran allergens is largely unknown, with only basic molecular functions being ascribed. Snake venoms have been extensively studied and it is interesting to compare their known functions with those unknown ones of Hymenoptera.

Venom allergens do not appear to have any unique antigenic properties, as a result it is likely that venom allergenicity occurs from the activation of accessory cells that secrete cytokines triggering the development of T helper and regulatory cells, in turn regulating the development of IgE-producing B cells in susceptible organisms [373]. Despite extensive research on allergens, it is still unknown what factors render proteins allergic, further evolutionary and molecular studies are required in order to unravel this allergenic mystery.

Given the almost ubiquitous occurrence of allergic reactions across mammals as a result of being stung by a bee, wasp or ant, it leaves the question why are hymenopteran venoms so allergenic? Proteins such as hyaluronidases and PLA_2 are present in various other venoms including snakes and centipedes. However, they rarely if ever cause the same allergic reaction that hymenopteran venoms induce. Is there an adjuvant present in the venom that may be influencing the allergic potential? Is it the structure or function of the allergenic protein? Or chemical and biological factors in the venom that cause this allergenic reaction? Studies looking at antibody response have suggested that low molecular weight hyaluronic acid polymers and oligomers in the skin released upon being stung may function as adjuvant to promote venom allergenicity [373]. However, this is yet to be explored further.

4.5. Conclusion

This study is the first of its kind to look at the evolutionary and molecular evolution of the major allergens found in hymenopteran venoms. We demonstrated that the major allergens present in hymenopteran venoms are evolving predominately under the influence of strong negative selection, while codon sites that experiencing episodic diversifying selection are concentrated on the surface suggesting a conservation of core amino acids. Functional testing is sorely needed in order to further understand the purpose of allergens. These

results emphasize the importance of understanding the molecular evolution, diversification, and phylogenetic histories of allergen components in hymenoptera.

CHAPTER 5

Conclusions and future directions

5.1. Research overview

The results presented in this thesis form a large-scale comparative examination of the aculeate (Aculeata, Hymenoptera) venom system. The selected species and their venoms were chosen so that they spanned a range of lifestyles and genera in order to examine interactions between life history and venom evolution. Aculeates have a uniquely derived venom-delivery apparatus and a fascinating diversity of social lifestyles within the group. Multiple aspects of the evolution of the aculeate venom system were investigated, painting a picture of insects that exhibit a diverse array of behaviour associated with distinctive and interesting venoms that diversified secondary to the evolution of the clade's sophisticated venom-delivery apparatus.

5.1.1. Solitary and social aculeate venom systems

While previous studies have been undertaken on a variety of aculeate species, very few have looked at the relationship between the social behaviour of the insects and their venom composition and venom-delivery apparatus. This thesis provides insight into how aculeate venoms co-evolved with their life history and behaviour. The research shows distinctive molecular compositions and activities consistent with different roles the venoms play. The venoms of both solitary and social aculeates are known to cause pain and tissue damage, however, there are many functions that are specific to their lifestyle. The venom of solitary aculeates is closely related to the venom of parasitic hymenoptera and is used offensively to capture prey and cause non-lethal paralysis. The venoms are thought to cause transient and/or permanent paralysis, most likely due to low molecular mass neurotoxins. In contrast the venoms in social aculeates have evolved in order to maximise their defensive potential by including toxins that cause pain and augment allergenic and immune responses. Pain is the most infamous defensive property of social aculeate venoms. A predator (or intruder) experiencing sudden and intense pain will in most cases respond by retreating, a behaviour advantageous to the insect. Through proteomics, mass spectrometry and activity testing it was found that social venoms have comparatively higher activities (mediated by peptidic toxins) that cause cell death and likely result in pain in the stung individual. They were also higher quantities of allergenic peptides identified through proteomics and mass spectrometry. These divergences in the venoms suggest that there is an increased evolutionary pressure on the venoms of social species in order to induce pain and damage.

The aculeus (stinger) of aculeate species was found to have unique morphological features that differ considerably within and between families. The aculei of solitary species lacked

substantial barbs, while the majority of aculei in social species possessed either serrations or barbs. The presence of barbs on the aculeus likely facilitates the phenomenon known as aculeus autonomy, the self-defence technique of self-amputation of the aculeus. The study also identified, for the first time, transition metals in the cuticle of the aculeus in aculeate species, with SEM-EDS point analyses showing that metals in the cuticle are exclusively found around the tip and edges of the aculeus. This likely suggests that the metals present play an adaptive functional role.

5.1.2. Evolution of aculeate allergens

In order to gain a better understanding of the aculeate venom system, the evolutionary forces that shape the toxins present in the venom need to be unravelled. Allergens play an important role in defence in hymenopteran insects and potentially confer the evolutionary advantage of learned avoidance in predators. Molecular evolutionary assessments revealed that the major allergens (PLA_1, PLA_2, hyaluronidase, acid phosphatase, serine protease and antigen 5) in hymenopteran venoms are evolving predominately under the influence of strong negative selection. The individual codon sites in the allergens were also studied and found to be under negative selection, likely in order to maintain the structural integrity of the molecule. A small number of codons on the surface of the allergen were identified as under the influence of episodic diversifying selection. This pattern of evolution is consistent with rapid accumulation of variation in the exposed residues seen in other venomous lineages, most likely allowing the allergens to adapt to any host immune response. It is possible that some of the codons conserved under negative selection are the site of IgE-binding epitopes. However, the functional activity of allergens in hymenoptera is largely unknown and they do not appear to have any unique antigenic properties. As a result, it is still undetermined what factors or sites render proteins allergenic.

Overall these findings highlight the role of negative and episodic selection in shaping allergens in hymenopteran venom. The influence of negative selection is most likely advantageous in ensuring the preservation of core amino acids that are potentially involved in allergenicity and toxicity.

5.1.3. Future Directions

The chapters in this thesis provide a comparative insight into the evolution of aculeate venom systems and in the context of their social behaviour. However, it also introduces a large number of unanswered questions and ideas for future studies. The presence of

transition metals in the aculeus was demonstrated, although whether or not these metals have any functional properties has not been explored. Further, while comparing solitary and social venoms, there was found to be a lack of sequenced and annotated solitary venom proteins, limiting the ability to match peptide sequence tags obtained by mass spectrometry to a database. The venoms of solitary species are still sorely understudied and addressing this deficit requires a combination of large-scale transcriptomic and proteomic studies in order to unravel their venom composition. Since many solitary wasps are specialists on certain prey/host species, it would be worthwhile investigating this characteristic and if it has an effect on how the venom composition evolves. Further, while the molecular functions of many peptides are known the biological functions remain elusive. Characterising the venom components at a functional level would greatly improve our understanding of venom interactions and facilitate the efficient investigation of aculeate venoms for biodiscovery and pharmacological purposes.

Venom allergens are very important components in aculeate venoms, contributing to an estimated 100 deaths by stinging hymenoptera per year. Venom allergens were demonstrated to be present in both solitary and social species and are likely important components of their defensive strategies. However, the molecular functions of allergens and the factors that cause the allergenic reaction are still unknown. Unravelling this mystery of allergens in aculeate venoms would be not only be interesting but also very useful, as this improved understanding would open the door to treating patients that are hypersensitive to not only aculeate venoms but also those whose have any IgE-related chronic diseases.

5.2. Conclusion

This thesis has provided evidence that solitary and social lifestyles of aculeates have resulted in a divergence in venom composition and apparatus. It also provides an investigation of the evolutionary forces shaping allergen evolution in venom. However, many unresolved questions remain. Hopefully this thesis provides a firm foundation on which future research can be established.

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Appendix I

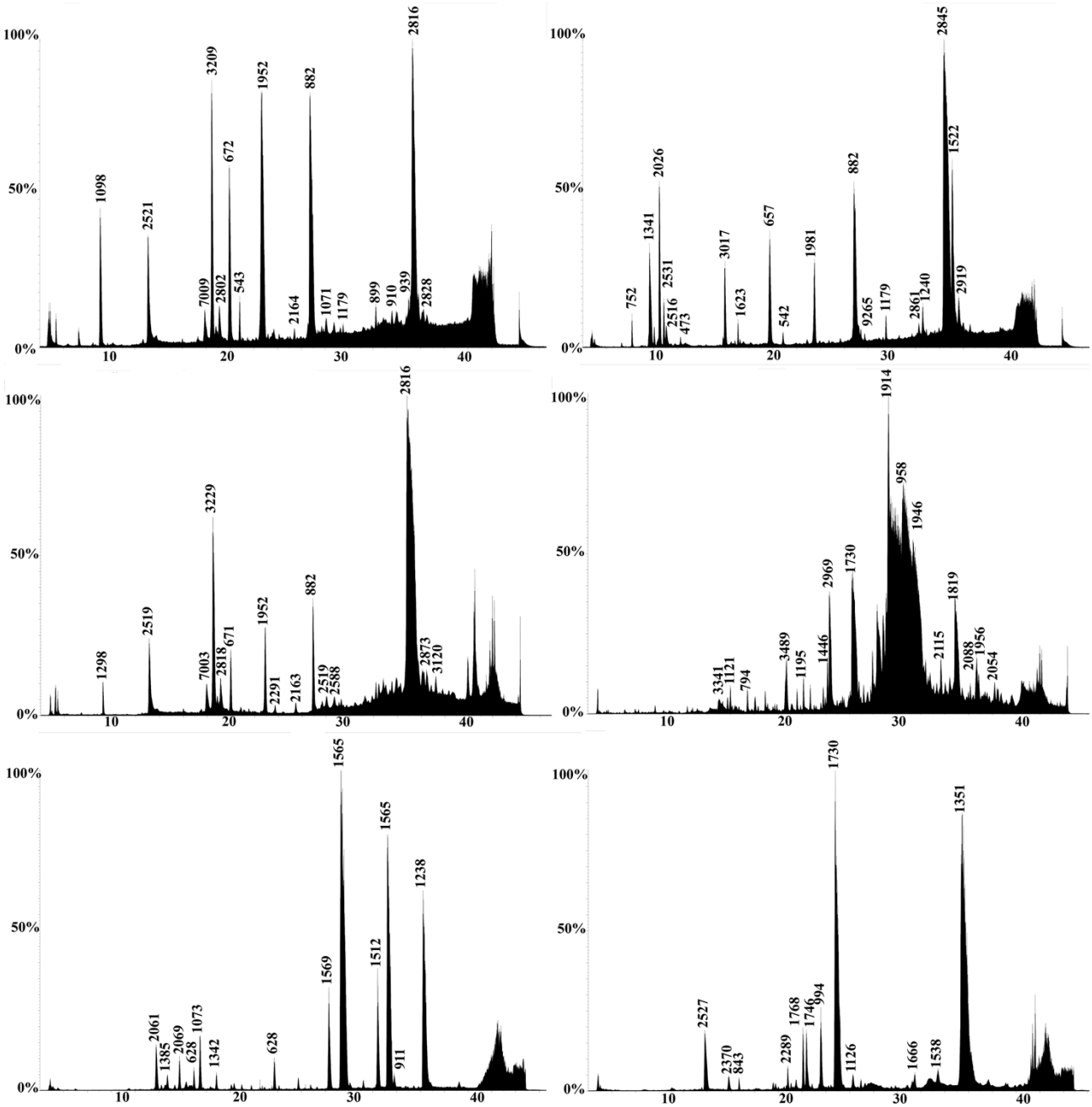

Figure S3.1. Representative LC-MS profiles of bee species A) *Apis cerana* B) *Apis dorsata* C) *Apis florea* D) *Bombus huntii* E) *Centris aethyctera* F) *Diadasia rinconis* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

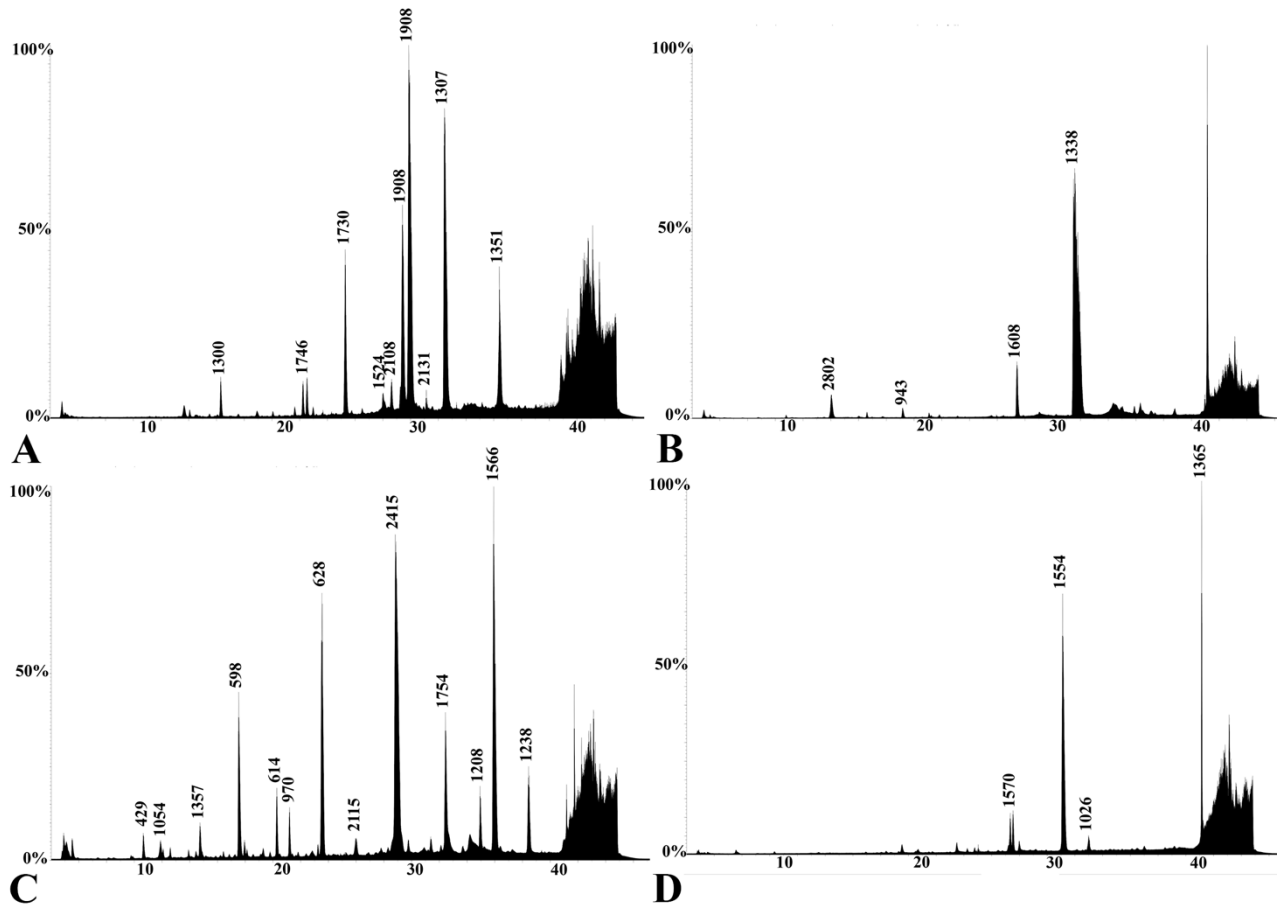

Figure S3.2. Representative LC-MS profiles of bee species A) *Crawfordapis* sp. B) *Xylocopa rufa* C) *Lasioglossum* sp. D) *Xylocopa veripuncta* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

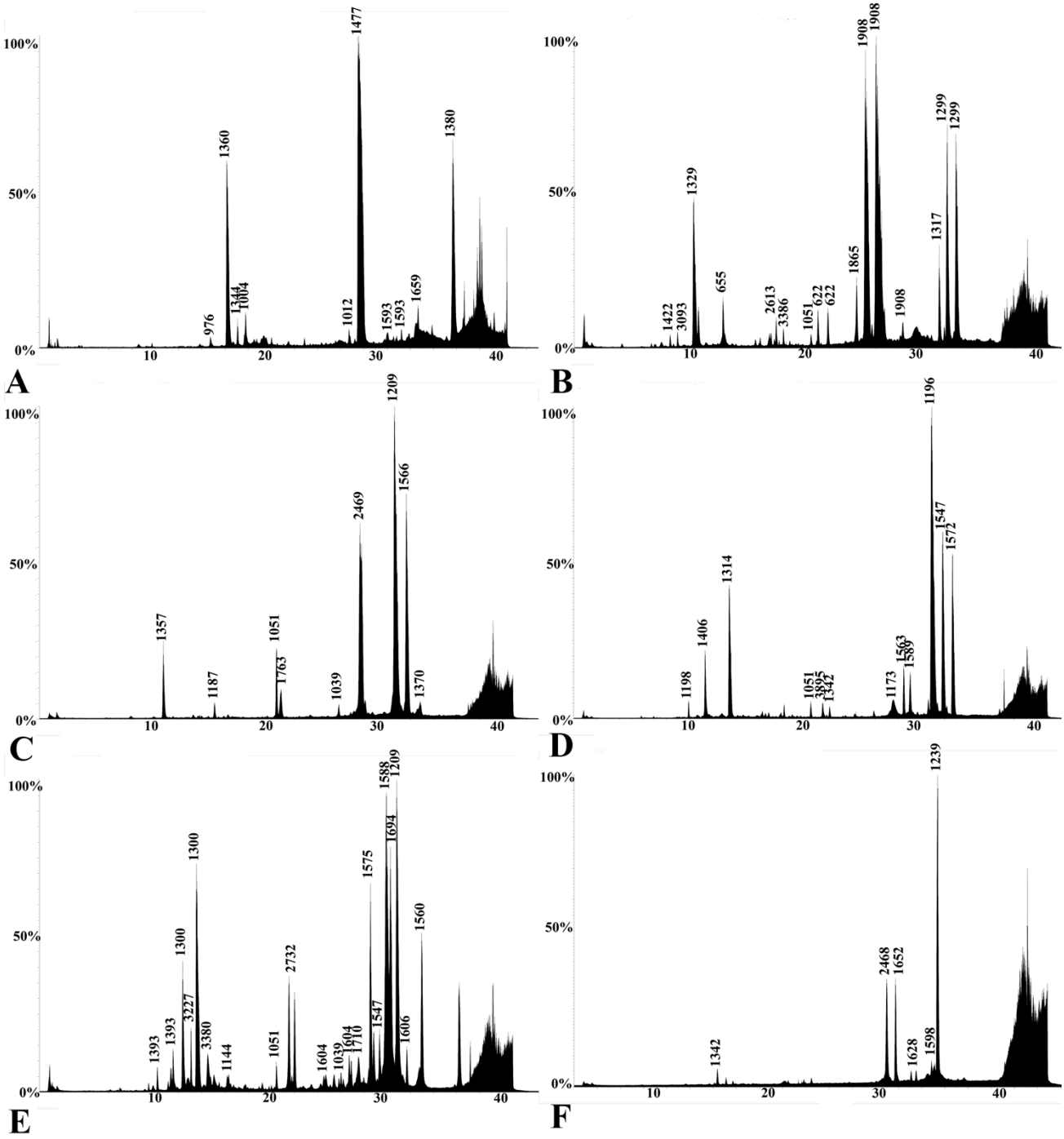

Figure S3.3. Representative LC-MS profiles of Vespidae species A) *Vespa mandarinia* B) *Vespa tropica* C) *Vespa luctuosa* D) *Vespa simillima* E) *Vespula pensylvanica* F) *Dolichovespula arenaria* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

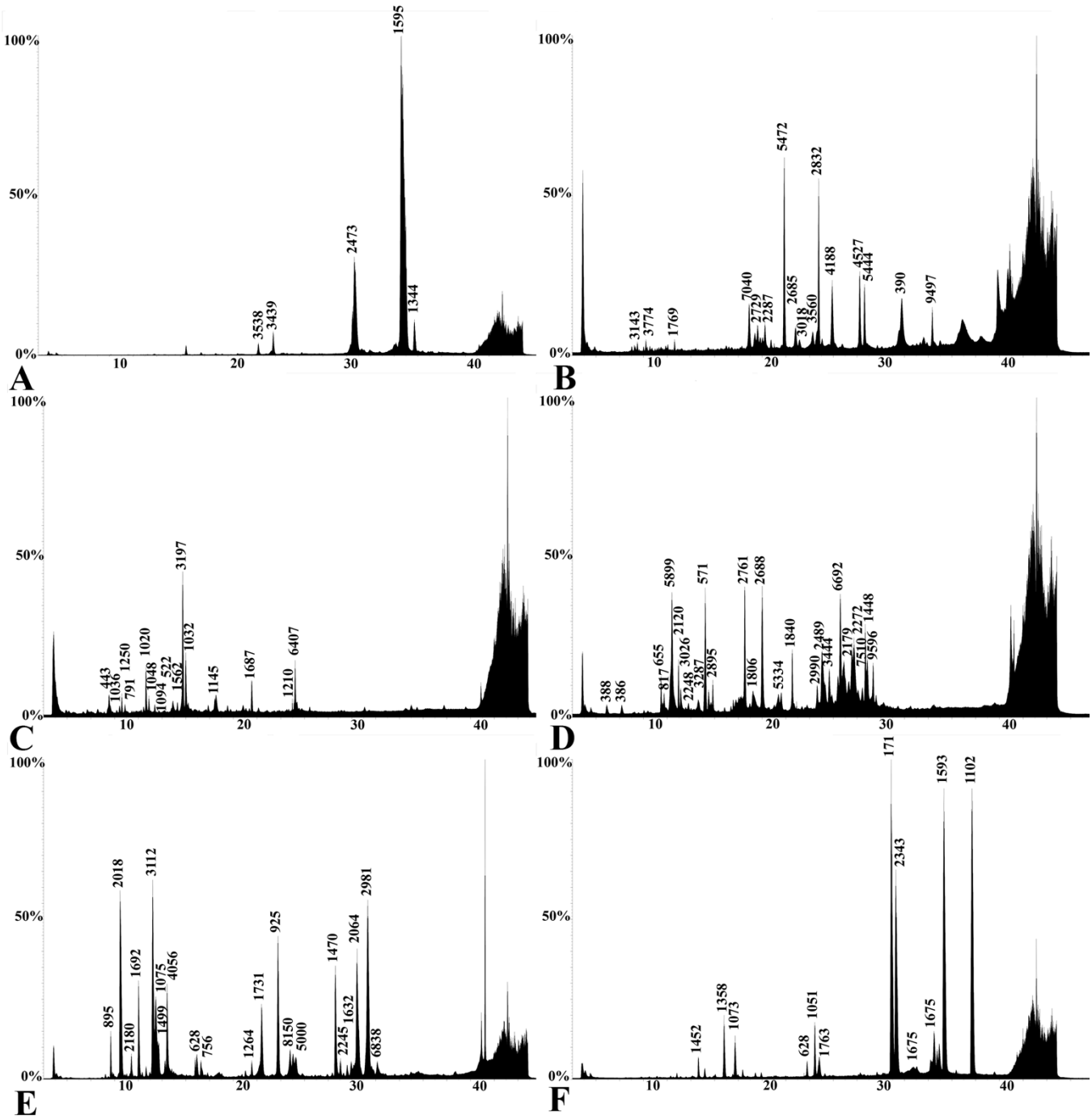

Figure S3.4. Representative LC-MS profiles of Vespidae species A) *Polybia rejecta* B) *Polistes flavus* C) *Polistes canadensis* D) *Polistes comanchus navajoe* E) *Parachartergus fraternus* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

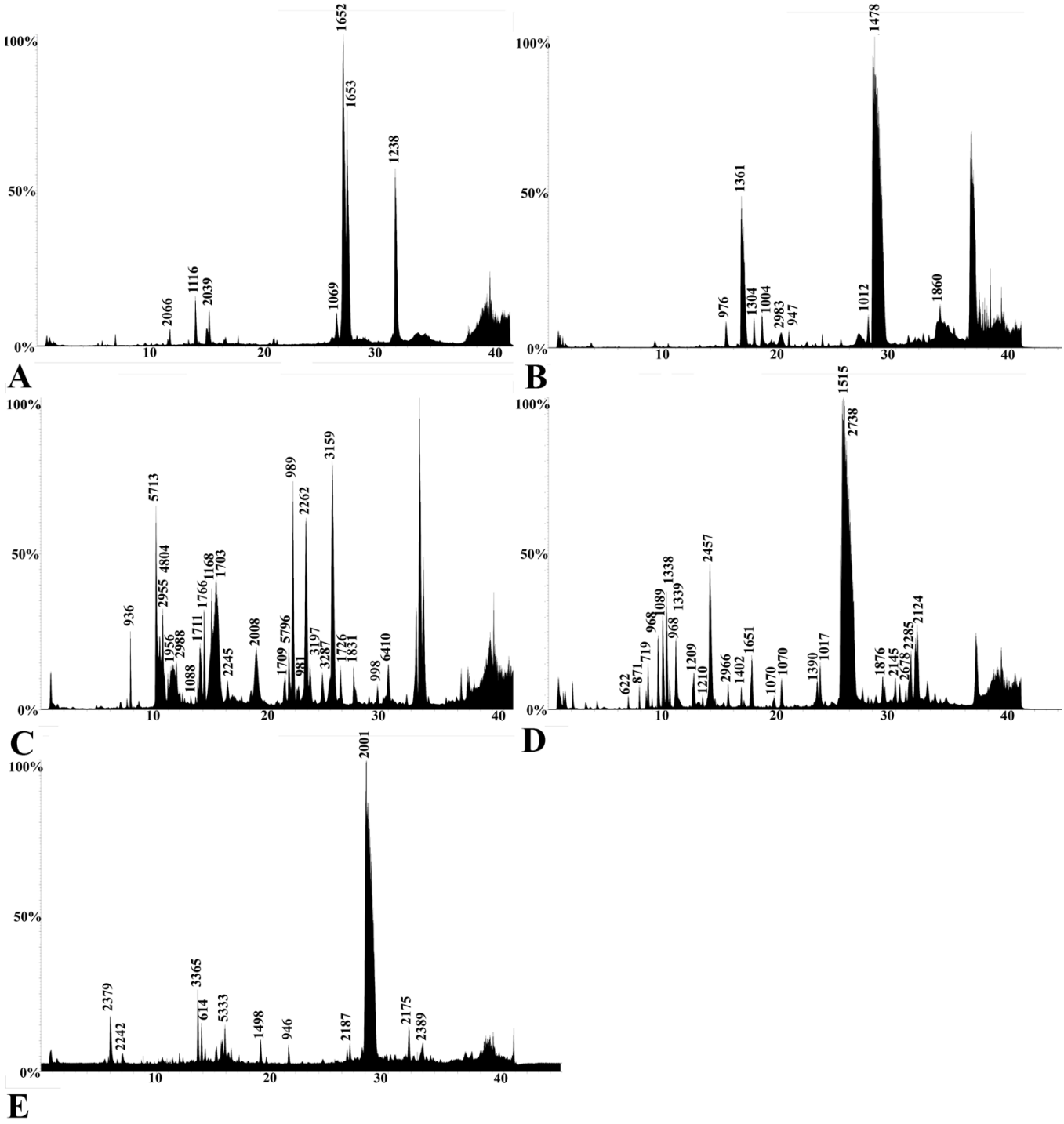

Figure S3.5. Representative LC-MS profiles of Vespidae species A) *Mischocyttarus flavitarsus* B) *Belonogaster juncea colonialis* C) *Brachygastra mellifica* D) *Polybia simillima* E) *Scollidae* sp. X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

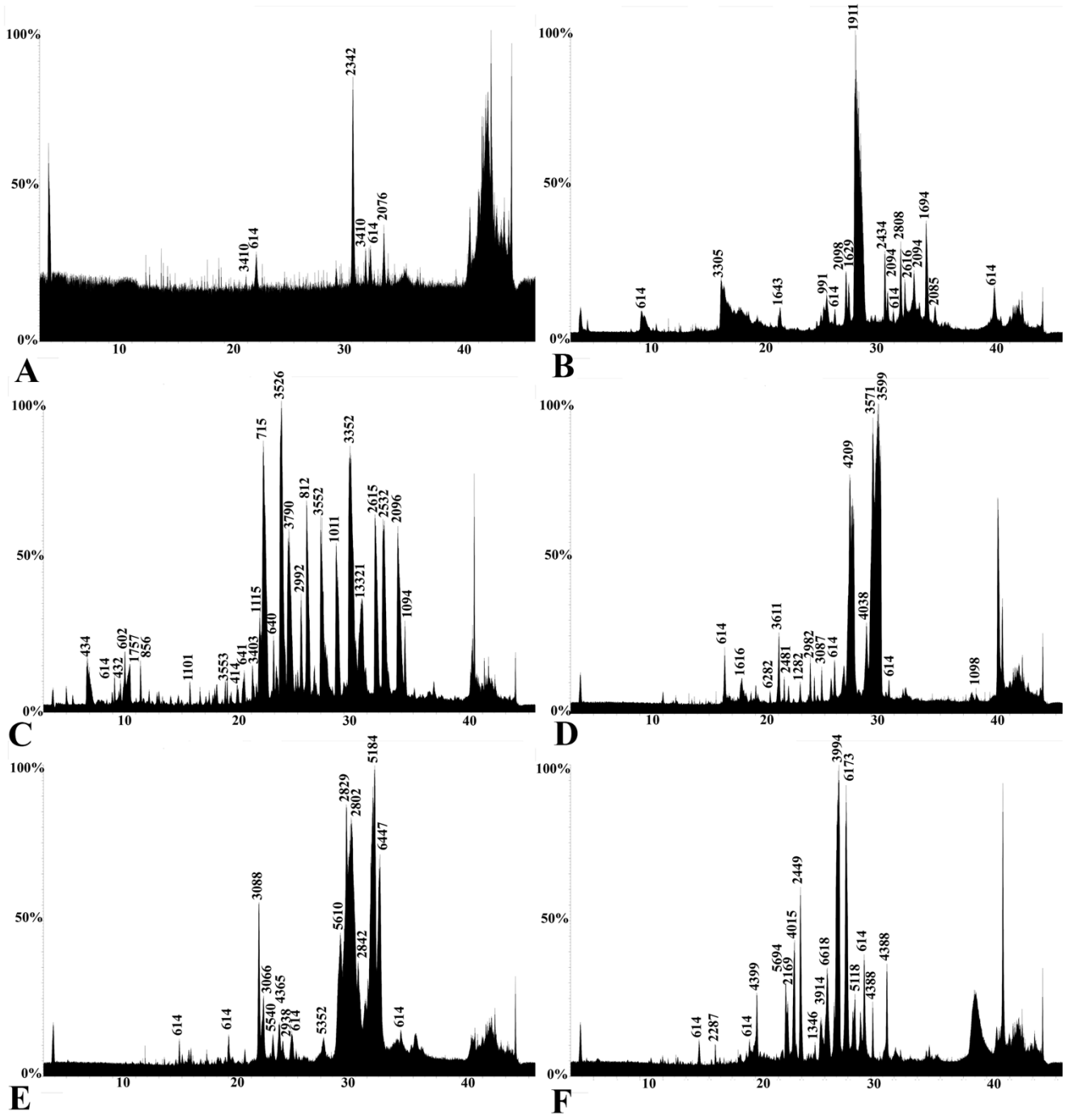

Figure S3.6. Representative LC-MS profiles of Vespidae species A) *Myrmecia tarsata* B) *Myrmecia pilosula* C) *Myrmecia simillima* D) *Myrmecia gulosa* E) *Myrmecia browningii* F) *Leptogenys* sp. X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

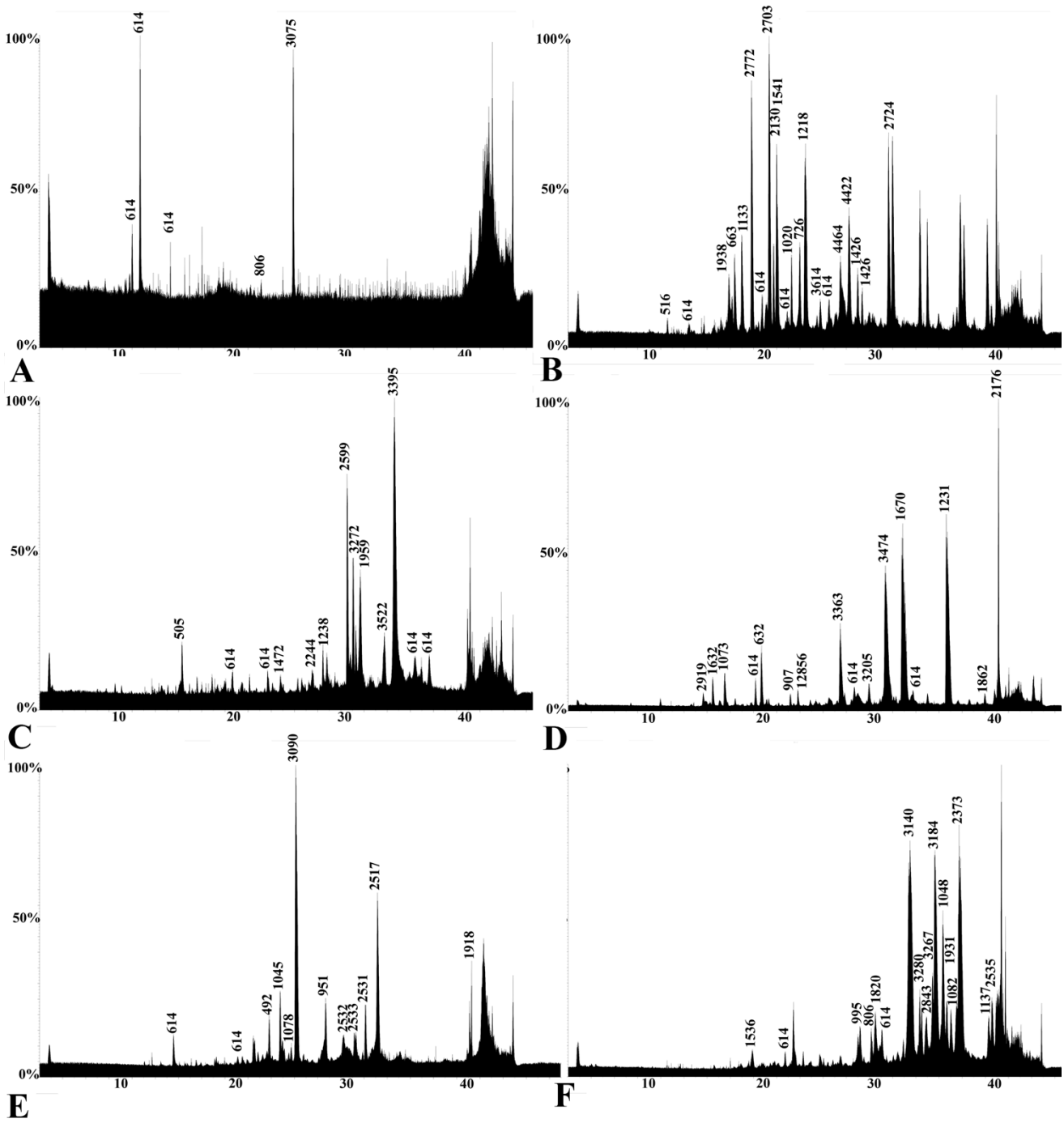

Figure S3.7. Representative LC-MS profiles of Vespidae species A) *Daceon* sp. B) *Megaponera analis* C) *Gnaptogenys* sp. D) *Ectatomma* sp. E) *Rhytidoponera metallica* F) *Ectatomma tuberculatum* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

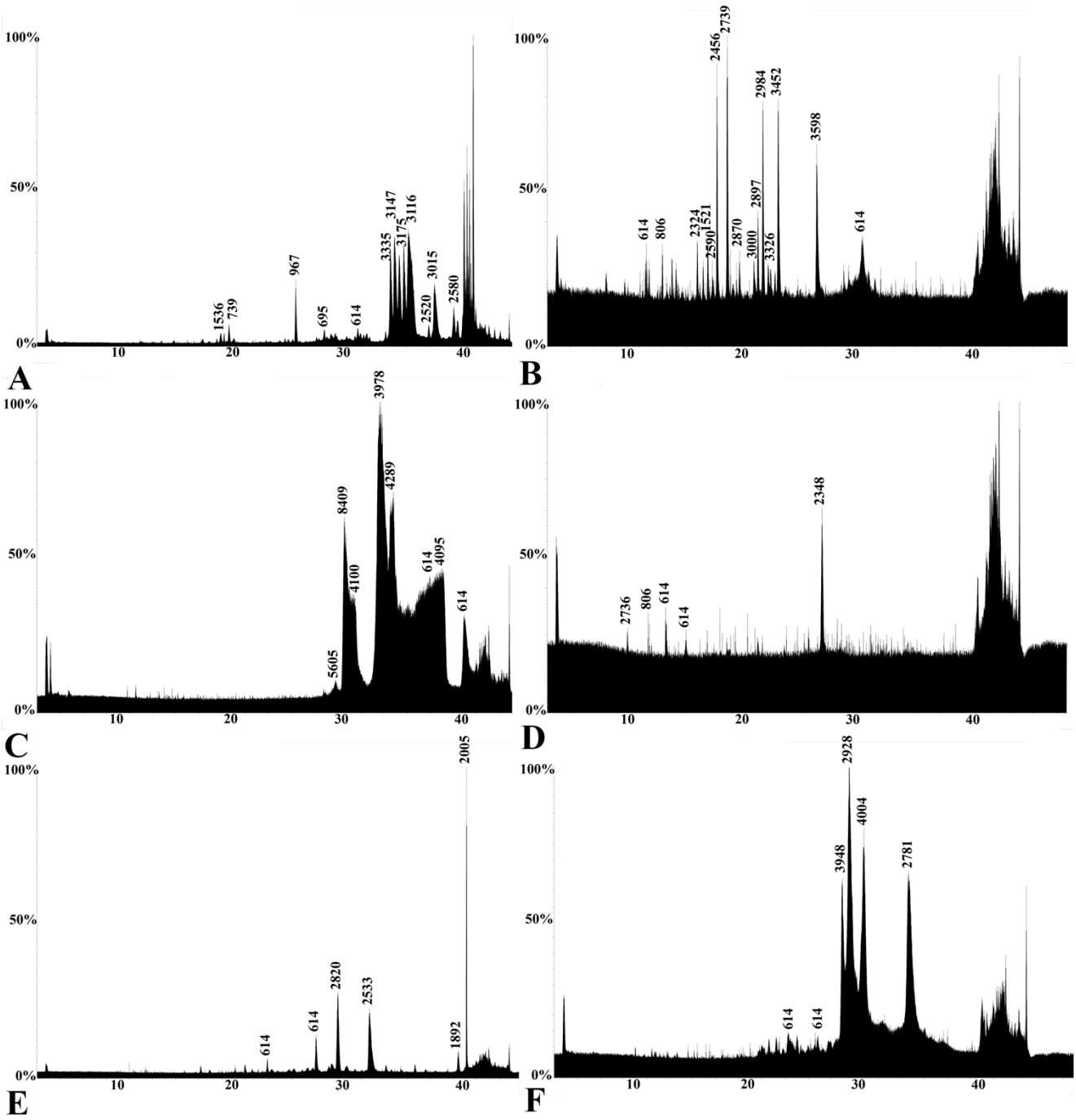

Figure S3.8. Representative LC-MS profiles of Vespidae species A) *Termitopone communtata* B) *Termitopone communtata* (Queen) C) *Opthalmopone* sp. D) *Pogonomyrmex maricopa* E) *Tetraponera* sp. F) *Pogonomyrmex rugosus* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

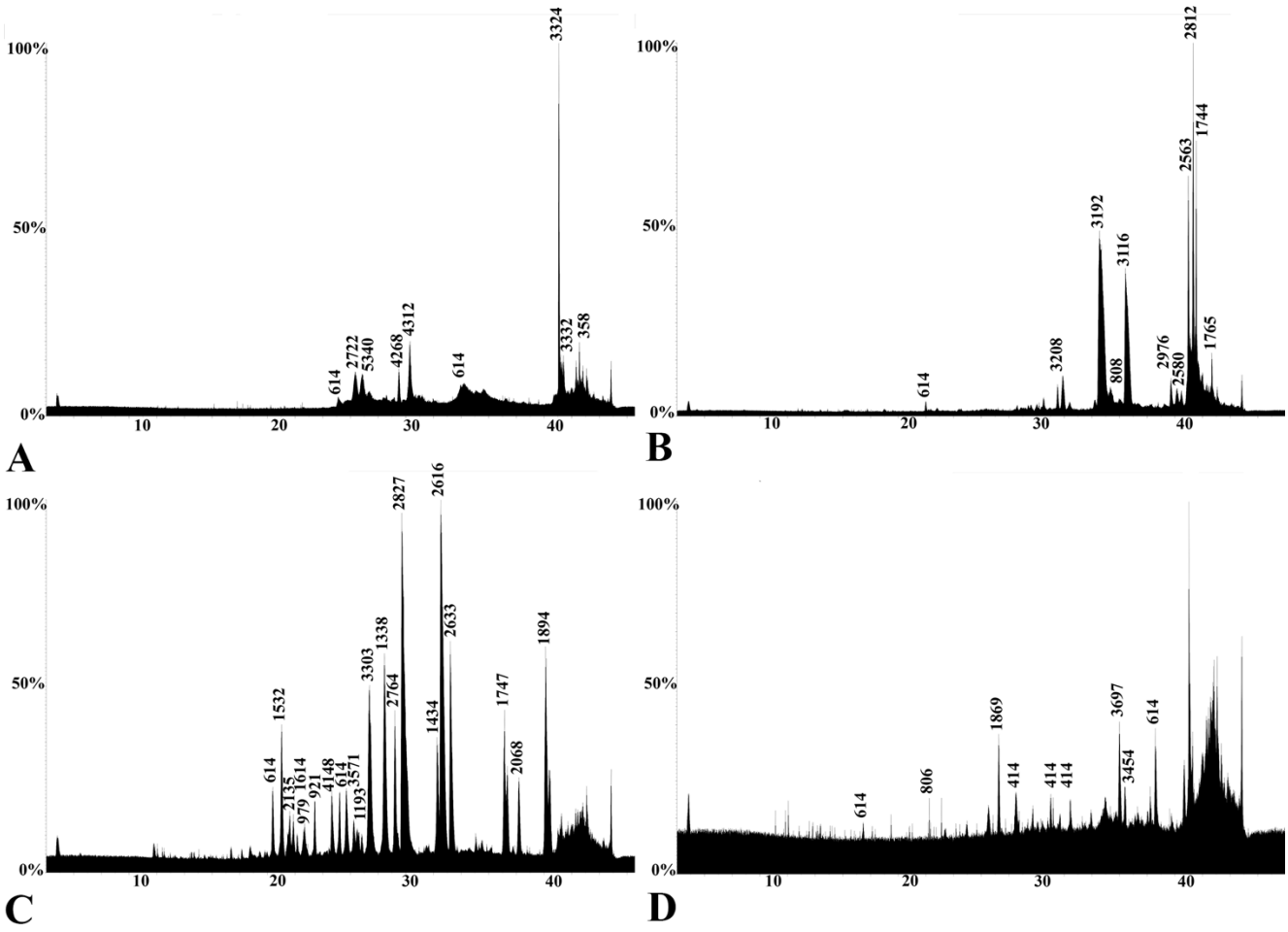

Figure S3.9. Representative LC-MS profiles of Vespidae species A) *Paraponera clavata* B) *Pogonomyrmex occidentalis* C) *Platythyrea lamellosa* D) *Streblognathus aethiopicus* X-axis is time (minutes); Y-axis is relative intensity (0–100%). Reconstructed mass in Daltons is shown above each peak.

STable 3.1. NFF venom cytotoxicity (%) ± SEM.

Species	5 ug	0.5 ug	AUC
Apis mellifera (European)	99.84±0.001	15.81±0.006	260.1
Apis mellifera (Africanised)	99.92±0.002	0.98±0.07	248.7
Apis andreniformis	95.07±0.008	30.91±0.01	283.5
Apis cerana	45.28±0.01	9.99±0.003	124.4
Apis dorsata	98.97±0.00	1.66±0.05	228.8
Apis florea	34.04±0.02	7.81±0.01	94.17
Bombus huntii	0.00	19.59±0.008	44.09
Bombus sonorus	56.38±0.02	18.24±0.007	167.9
Crawforapis sp.	0.00	0.00	3.655
Centris aethyctera	12.72±0.16	0.00	28.73
Diadasia rinconis	2.36±0.07	0.00	29.43
Lasioglossum sp.	10.71±0.05	8.75±0.05	43.78
Peponapis pruinosa	0.00	6.80±0.06	22.46
Xylocopa rufa	3.56±0.12	0.00	20.36
Xylocopa angustior	28.44±0.04	1.77±0.10	67.83
Xylocopa californica	0.00	8.55±0.02	23.44
Xylocopa varipuncta	5.29±0.11	3.53±0.07	22.24
Agelaia myrmecophila	25.51±0.08	26.19±0.01	116.3
Belonogaster juncea colonialis	23.11±0.05	34.36±0.01	167.6
Brachygastra mellifica	18.83±0.13	31.12±0.01	112.4
Dolichovespula arenaria	20.69±0.23	18.56±0.05	88.36
Dasymutllia chiron	6.76±0.15	15.41±0.03	63.66
Dasymutllia gloriosa	39.32±0.14	66.67±0.01	238.5
Dasymutllia klugii	28.86±0.08	36.49±0.02	147
Mischocyttarus flavitarsus	16.49±0.18	20.05±0.03	96.44
Megapolistes sp.	25.64±0.04	11.15±0.01	89.08
Polistes canadensis	43.09±0.03	22.47±0.01	147.5
Polistes comanchus navajoe	41.03±0.04	24.04±0.04	151.9
Polistes dorsalis	45.53±0.06	40.22±0.01	192.9
Polistes flavus	41.74±0.05	25.22±0.02	184
Parachartergu fraternus	36.99±0.06	44.12±0.01	182.5
Polistes major castaneocolor	27.91±0.07	1.94±0.3	98.7
Polybia rejecta	23.25±0.12	17.23±0.05	116.7
Polybia simillima	6.09±0.13	31.13±0.01	68.63
Ropalida sp.	47.34±0.06	26.11±0.03	166.7
Synoeca septentrionalis	17.47±0.11	28.16±0.06	113.8
Scollidae sp.	15.09±0.09	22.79±0.02	85.22
Stictia sp.	14.75±0.05	7.43±0.02	63.46

Vespula pensylvanica	35.59±0.08	17.28±0.03	123.7
Vespa tropica	41.74±0.06	28.00±0.02	156.9
Vespula vulgaris	25.22±0.16	4.29±0.03	92.54
Vespa mandarinia	32.2±0.05	25.46±0.04	135.3
Vespa simillima	36.36±0.08	34.97±0.03	161.7
Vespa luctuosa	29.23±0.14	8.43±0.07	84.73
Brachyponera sennaarensis	51.66±0.16	83.03±0.03	302.8
Daceton sp	37.32±0.14	64.67±0.07	229.5
Diacamma sp	1.69±0.25	5.39±0.02	34.66
Ectatomma tuberculum	98.22±0.01	100	442
Leptogenys sp	13.01±0.24	7.78±0.03	51.7
Megaponera analis	6.49±0.24	1.59±0.03	35.62
Myrmecia browningii	18.09±0.23	52.46±0.07	162.2
Myrmecia gulosa	45.98±0.24	89.21±0.03	202.6
Myrmecia nigripes	14.80±0.03	0.14±0.14	130.6
Myrmecia pilosula	100	100	448.8
Myrmecia rufinodis	43.25±0.20	64.36±0.05	242.1
Myrmecia simillima	75.39±0.11	87.88±0.02	366.7
Myrmecia tarsata	61.45±0.24	81.96±0.06	322.2
Neoponera villosa	19.49±0.16	38.57±0.06	139.7
Odontomachus sp	6.69±0.09	0	22.34
Odontoponera sp	3.11±0.28	7.69±0.02	45.3
Opthalmopone sp	0	0	8.846
Pachycondyla crassinoda	23.66±0.20	48.70±0.06	206.1
Paltothyreus tarsatus	15.09±0.24	36.20±0.07	162.8
Paraponera clavata	44.04±0.14	47.56±0.02	102.8
Platythyrea lamellosa	16.63±0.09	29.04±0.01	112
Platythyrea strigulosa	5.10±0.27	24.00±0.01	139.2
Pogonomyrmex maricopa	5.43±0.16	19.39±0.07	49.63
Pogonomyrmex occidentalis	20.23±0.05	25.25±0.11	69.51
Pogonomyrmex rugosus	0	0	134.4
Streblognathus aethiopicus	10.01±0.16	39.83±0.08	142.3
Termitopone commutata	21.89±0.03	54.15±0.08	181
Termitopone commutata Queen	11.95±0.13	11.63±0.01	65.1
Tetraponera sp	29.57±0.09	66.14±0.06	214.3

STable 3.2. MM96L venom cytotoxicity (%) ± SEM.

Species	5 ug	0.5 ug	AUC
Apis mellifera (European)	100.00	18.91±0.02	267.6
Apis mellifera (Africanised)	100.00	16.17±0.03	261.2
Apis andreniformis	99.47±0.004	21.73±0.02	272.5
Apis cerana	46.65±0.02	0.00	105
Apis dorsata	99.98±0.00	23.72±0.02	278.3
Apis florea	15.92±0.01	0.00	35.84
Bombus huntii	65.73±0.03	0.00	147.9
Bombus sonorus	46.52±0.05	0.00	104.7
Crawforapis sp.	42.86±0.19	17.90±0.11	119.6
Centris aethyctera	20.18±0.20	9.95±0.04	39.16
Diadasia rinconis	16.88±0.06	5.08±0.05	54.72
Lasioglossum sp.	28.84±0.04	8.03±0.03	86.8
Peponapis pruinosa	14.59±0.02	7.04±0.02	48.67
Xylocopa rufa	0.00	5.87±0.04	16.07
Xylocopa angustior	30.99±0.03	13.49±0.02	100.1
Xylocopa californica	15.56±0.07	0.40±0.04	39.93
Xylocopa varipuncta	47.15±0.17	11.71±0.03	77.23
Agelaia myrmecophila	10.98±0.03	3.40±0.04	51.69
Belonogaster juncea colonialis	83.42±4.14	14.38±0.07	226
Brachygastra mellifica	12.87±0.03	24.82±0.07	84.8
Dolichovespula arenaria	13.52±0.03	26.10±0.14	116.1
Dasymutllia chiron	12.93±0.04	15.57±0.09	68.74
Dasymutllia gloriosa	16.54±0.04	19.87±0.07	91.34
Dasymutllia klugii	59.26±0.05	12.32±0.05	168.8
Mischocyttarus flavitarsus	4.28±0.03	9.82±0.08	44.77
Megapolistes sp.	31.08±0.05	1.02±0.05	91.95
Polistes canadensis	14.49±0.06	21.13±0.06	84.23
Polistes comanchus navajoe	27.50±0.09	12.90±0.03	99.52
Polistes dorsalis	8.6±0.05	10.75±0.06	55.3
Polistes flavus	15.59±0.03	9.39±0.06	52.9
Parachartergu fraternus	69.71±0.21	22.07±0.07	210.3
Polistes major castaneocolor	21.75±0.04	16.44±0.05	95.08
Polybia rejecta	31.85±0.07	15.77±0.06	109.3
Polybia simillima	1.44±0.03	2.71±0.05	28.98
Ropalida sp.	29.31±0.04	13.44±0.02	105.6
Synoeca septentrionalis	47.76±0.16	14.05±0.08	148.1
Scollidae sp.	5.14±0.03	0.34±0.05	30.11
Stictia sp.	11.95±0.04	0	44.17

Vespula pensylvanica	27.02±0.08	2.39±0.14	71.66
Vespa tropica	14.30±0.02	25.23±0.07	90.1
Vespula vulgaris	20.35±0.09	6.29±0.05	69.82
Vespa mandarinia	18.21±0.05	36.08±0.07	122.1
Vespa simillima	12.04±0.05	21.67±0.05	75.89
Vespa luctuosa	18.75±0.04	5.51±0.07	70.85
Brachyponera sennaarensis	99.53±0.006	95.32±0.03	438
Daceton sp	62.56±0.04	66.47±0.10	290.3
Diacamma sp	3.71±0.02	6.59±0.04	23.2
Ectatomma tuberculum	100.39±0.002	76.39±0.26	396.6
Leptogenys sp	10.78±0.03	77.97±0.07	199.7
Megaponera analis	7.04±0.04	27.83±0.19	103.2
Myrmecia browningii	79.71±0.11	56.28±0.14	314.1
Myrmecia gulosa	99.78±0.003	60.45±0.27	360.2
Myrmecia nigripes	48.83±0.29	63.14±0.18	252.5
Myrmecia pilosula	99.91±0.002	99.38±0.004	447.7
Myrmecia rufinodis	95.66±0.01	76.01±0.11	386.3
Myrmecia simillima	100.07±0.004	97.17±0.02	442.5
Myrmecia tarsata	99.67±0.005	99.84±0.003	447.3
Neoponera villosa	79.85±0.04	8.23±0.05	225.5
Odontomachus sp	6.08±0.05	3.59±0.05	28.08
Odontoponera sp	5.25±0.04	25.31±0.13	90
Opthalmopone sp	0.88±0.01	40.08±0.20	93.65
Pachycondyla crassinoda	84.97±0.07	18.27±0.13	279
Paltothyreus tarsatus	25.27±0.03	18.59±0.09	245.5
Paraponera clavata	62.71±0.03	60.47±0.12	130.4
Platythyrea lamellosa	31.54±0.12	26.44±0.04	234.7
Platythyrea strigulosa	13.05±0.05	31.22±0.21	281.6
Pogonomyrmex maricopa	57.89±0.02	36.27±0.19	169.4
Pogonomyrmex occidentalis	99.94±0.004	25.53±0.04	100.8
Pogonomyrmex rugosus	38.37±0.11	36.93±0.10	100.2
Streblognathus aethiopicus	84.89±0.02	31.81±0.15	262.6
Termitopone commutata	55.02±0.17	23.19±0.05	176.7
Termitopone commutata Queen	7.81±0.04	17.73±0.07	57.92
Tetraponera sp	90.40±0.09	53.98±0.12	324.9